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(Agro Paris Tech)

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présentée et soutenue publiquement par

Alexandra DUVERGER

Le 12 décembre 2007

Régulation des réponses immunes des muqueuses par les dérivés de la toxine œdémateuse de l'anthrax.

Regulation of mucosal immune responses by anthrax edema toxin derivatives.

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Résumé substantiel

Contrairement aux vaccins injectables, les vaccins des muqueuses s'administrent facilement et s'accompagnent rarement d'effets secondaires. De plus, comme ils favorisent de fortes réponses humorale et à médiation cellulaire au niveau des muqueuses, seuls les vaccins des muqueuses offrent une protection optimale contre les pathogènes qui souvent empruntent les portes d'entrée telles que la bouche, le nez, le tractus génital ou rectal ou la peau pour envahir l'hôte. Cependant, des difficultés pour developper des adjuvants des muqueuses efficaces et sans effets secondaires ont ralenti la mise sur le marché de nouveaux vaccins administrables par voie des muqueuses. Les entérotoxines, comme la toxine cholérique de *Vibrio cholerae* (CT), sont des adjuvants des muqueuses très puissants. Néanmoins, leur toxicité exclut toute utilisation dans des vaccins chez l'homme. En tant que modèles expérimentaux, ces entérotoxines ont permis de souligner le rôle de l'activité enzymatique et l'importance du type de récepteurs auxquels les adjuvants se fixent et donc d'élucider certaines propriétés des adjuvants et leur toxicité inhérente.

Suite à l'observation que l'administration nasale de doses subléthales de la toxine œdémateuse de *Bacillus anthracis* (EdTx) n'inhibait pas mais plutôt augmentait les réponses immunes à un vaccin nasal protéique contenant CT comme adjuvant, nous nous sommes demandés si EdTx pouvait agir comme adjuvant des muqueuses. Nous avons démontré qu'EdTx, qui se fixe aux récepteurs des toxines de l'anthrax, est un adjuvant capable de promouvoir une immunité systémique et des muqueuses à des vaccins protéiques administrés par voie nasale, et génère une gamme de réponses immunes différente de CT, qui elle, se fixe aux gangliosides. Contrairement à CT, les dérivés d'EdTx ne ciblent pas les tissus du système nerveux central après administration nasale. Nous avons montré qu'EdTx améliore les fonctions de présentation de l'antigène par les macrophages, mais ne stimule pas la production de cytokines pro-inflammatoires. NGF (Nerve Growth Factor), un facteur inné qui a un rôle dans l'induction de l'immunité des muqueuses par CT, n'affecte pas les réponses immunes des muqueuses engendrées par EdTx *in vivo*, mais est un élément-clef dans la production d'IgA par des lymphocytes B stimulés par EdTx ou CT *in vitro*. Récemment, il a été rapporté que CT peut promouvoir une réponse immune dans le tractus intestinal ou même génital après immunisation transcutanée. Nous avons voulu tester l'efficacité de l'adjuvant EdTx par les voies transcutanée et sublinguale, voies très attractives pour la délivrance de vaccins. Il est apparu qu'EdTx est

capable d'induire des réponses immunes systémiques par ces deux voies, mais que seule la voie sublinguale permet le développement d'immunoglobulines A dans les muqueuses.

Abstract

Mucosal vaccines can be easily administered and favor humoral and cell-mediated immunity at mucosal sites. Thus, mucosal vaccines most likely offer optimal protection against pathogens that invade the host via mucosal surfaces. However, licensing of new mucosal vaccines is hampered by the lack of safe and efficacious mucosal adjuvants. The enterotoxin cholera toxin (CT) is a powerful adjuvant but its toxicity precludes its use in humans. As experimental models, enterotoxins have highlighted the importance of the enzymatic activity and targeted receptors in their adjuvanticity. Our work stems from the observation that sublethal doses of *Bacillus anthracis* edema toxin (EdTx) do not inhibit immune responses to nasal vaccines containing CT as adjuvant. We have now shown that EdTx derivatives represent a new class of adjuvants, which promote mucosal and systemic immune responses to vaccines given via multiple routes. We demonstrated that EdTx is capable of inducing systemic and mucosal immunity to protein vaccines administered nasally. Unlike the ganglioside-binding CT, EdTx, which bind the anthrax toxin receptors, do not target central nervous system tissues after nasal delivery. Furthermore, the innate factor nerve growth factor, which plays a role in the induction of mucosal immune responses by CT, did not affect the adjuvant activity of EdTx *in vivo*. We have established that the adjuvant activity of EdTx involves the enhancement of antigen presenting cell functions. Finally, we showed that EdTx is an effective adjuvant via both the transcutaneous and sublingual routes, although it could give rise to mucosal IgA responses only after sublingual immunization.

Key words:

Anthrax, toxins, adjuvant, nasal, cutaneous, sublingual, mucosal, immunity, IgA

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Résumé

Les vaccins des muqueuses s'administrent facilement et favorisent une immunité humorale et cellulaire au niveau des muqueuses. Ainsi, ils offrent une protection optimale contre les pathogènes envahissant par les muqueuses. Cependant, la mise sur le marché de nouveaux vaccins des muqueuses est entravée par le manque d'adjuvants des muqueuses efficaces et sans effets secondaires. La toxine cholérique (CT) est une entérotoxine à fort pouvoir adjuvant mais sa toxicité empêche son utilisation chez l'homme. En tant que modèle expérimental, elle a permis de comprendre l'importance de l'activité enzymatique et des récepteurs dans l'adjuvanticité. Notre travail se base sur l'observation que des doses sublétales de la toxine œdémateuse de *Bacillus anthracis* (EdTx) n'inhibent pas la réponse immune à des vaccins « nasaux » contenant CT comme adjuvant. Nous avons montré que les dérivés EdTx représentent une nouvelle classe d'adjuvants qui donnent des réponses systémiques et des muqueuses à des protéines vaccinales administrées par de multiples voies, notamment nasale. Contrairement à CT qui se fixe aux gangliosides, EdTx se fixe aux récepteurs des toxines de l'anthrax et ne cible pas les tissus du système nerveux central après administration nasale. Le facteur inné nerve growth factor intervient dans les réponses des muqueuses induites par CT mais n'affecte pas l'adjuvanticité d'EdTx *in vivo*. L'activité adjuvante d'EdTx implique aussi l'augmentation des fonctions de présentation de l'antigène. Enfin, nous avons montré qu'EdTx est un adjuvant efficace par les voies transcutanée et sublinguale, bien que les IgA des muqueuses ne soient induits qu'après immunisation sublinguale.

Mots-clés :
Anthrax, toxines, adjuvant, nasal, cutané, sublingual, muqueux, immunité, IgA

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Abbreviations

2 Abbreviations

Ab _____Antibody
Ag _____Antigen
CLN _____Cervical lymph nodes
CMI _____Cell-mediated immunity
CNS _____Central nervous system
CT _____Cholera toxin
CTb _____Binding subunit of CT
DC _____Dendritic cell
ECs _____Epithelial cells
EdTx _____*Bacillus anthracis* edema toxin (wild-type PA plus wild-type EF)
EdTx S414N ___Wild-type PA plus EF S414N
EF _____*Bacillus anthracis* edema factor (wild-type)
EF S414N _____Mutant of EF with 10% residual enzymatic activity
FAE _____Follicle-associated epithelium
GALT _____Gut-associated lymphoid tissue
GI _____Gastrointestinal
i.p. _____Intra-peritoneal
IFN-α/β/γ_____Interferon alpha/ beta/ gamma
IL _____Interleukine
LeTx _____*Bacillus anthracis* lethal toxin (wild-type PA plus wild-type LF)
LF _____*Bacillus anthracis* lethal factor (wild-type)
LP _____Lamina propria
MALT _____Mucosa-associated lymphoid tissues
MHC _____Major histocompatibility complex
MLN _____Mesenteric lymph nodes
MØ _____Macrophage
NALT _____Nasal-associated lymphoid tissue
NGF _____Nerve growth factor
NK cells _____Natural killer cells
OB _____Olfactory bulbs
ON/E _____Olfactory nerves/epithelium
OVA _____Ovalbumin
PA _____Wild-type PA
PA _____*Bacillus anthracis* protective antigen
PA dFF E308D _Mutant of PA unable to translocate EF or LF to the cytosol
PA-U7 _____Mutant of PA lacking the furin cleavage site
PBS _____Phosphate-buffered saline
pIgR _____Polymeric Ig receptor
PP _____Peyer's patch
SED _____Sub-epithelial dome
SIgA _____Secretory-IgA Abs
TCI _____Transcutaneous immunization
TGF-α/β_____Transforming growth factor alpha/ beta
Th _____T helper
TLR _____Toll-like receptor
wt_____Wild-type

Introduction

3 Introduction

Direct blood-borne infections are rare and only occur through injections, transfusions and bites. Most human pathogens enter the body through the mucosal surfaces, mainly the gastrointestinal, genitourinary and respiratory tracts. Cold viruses, influenza, food poisoning agents, tuberculosis, sexually transmitted diseases, cholera, diphtheria and plague are just a few examples. While it is widely accepted that provision of appropriate immunity at mucosal surfaces would be of great benefit for host immunity, only a few current vaccines specifically target the mucosal immunity. Vaccines delivered by mucosal (i.e., oral, nasal or rectal) routes most efficiently promote immunity in both mucosal tissues and the systemic compartment and thus, for the last two decades, great efforts are being undertaken to make mucosal immunization the benchmark method for mass immunization. These efforts include the development and characterization of adjuvants, which could be added to mucosal vaccines and enhance the magnitude and / or the quality of systemic and mucosal immune responses to co-inoculated vaccine antigens.

3.1 The mucosal immune system

3.1.1 The mucosal surfaces

Mucous membranes cover epithelial surfaces and comprise the linings of the digestive, the respiratory and the urogenital systems as well as eye conjunctiva, the inner ear and the ducts of all exocrine glands (salivary, gastric and sweat glands, prostate, liver). Overall in human, they cover the equivalent of one and a half tennis courts or a surface area of approximately 400m² [1]. The epithelium of mucosal surfaces is protected by a number of mechanisms including the mucus, antibodies, lipids and other non-mucin factors, such as antimicrobial peptides or defensins [2]. The mucosa hosts and selectively regulates an abundant microbiota and antigens. The interactions with commensal microbes and food antigens require the integrity of the mucosal barrier to be preserved. In the event the homeostasis is altered and protective immunity is needed, the epithelial cells lining the mucus membrane will help release secretory IgA (SIgA) antibodies, which are the hallmark of mucosal immunity.

3.1.2 Compartimentalization of the mucosal immune system

Highly sophisticated immune mechanisms help maintain the integrity of the mucosal surfaces and prevent the dissemination of pathogens and allergens in the (quasi) sterile systemic compartment. Thus, in addition to the innate mechanisms described above, mucosal surfaces defense arsenal involves well-orchestrated antigen-specific adaptive immune mechanisms. The later is possible by the accumulation in or the transition of immune cells between inductive sites and effector sites. Inductive sites for mucosal immunity consist in defined lymphoid structures, or mucosa-associated lymphoid tissues (MALT), which lack afferent lymphatics, as well as local draining lymph nodes. The effector sites constitute more diffuse lymphoid tissues, including the lamina propria (LP) of various mucosae, the stroma of exocrine glands and surface epithelia. In addition, specific homing receptor / addressin couples orchestrate homing of effector cells from inductive sites to distinct effector sites.

3.1.2.1 Induction of mucosal immunity in the MALT

Gut-associated lymphoid tissue (GALT) comprise the Peyer's patches (PP), the appendix, solitary lymphoid nodules, crytopatches [3] and isolated lymphoid follicles [4]. In humans, inductive sites of the upper respiratory tract, and oral / nasal cavities are represented by the unpaired nasopharyngeal tonsil or adenoids, the paired palatine tonsils and other smaller lymphoid structures of Waldeyer's pharyngeal ring, collectively named nasal- or nose-associated lymphoid tissue (NALT). While tonsils are not present in rodents, NALT structures exist and are localized on the cartilaginous soft palate (Figure 1). The GALT has been the object of extensive studies and often serves to describe how immune responses are initiated in inductive sites. Intestinal antigens can be taken across the epithelial cell layer through specialized membrane/microfold or M cells located in the follicle-associated epithelium (FAE) of the PP. Antigens are then captured by APCs of the subepithelial dome (SED), which are mostly dendritic cells (DCs). This event triggers DC to mature and migrate to the interfollicular region (IFR) of PP, and later MLN, where they present antigens to T cells. Those T cells can in turn interact with adjacent B cells in B cell follicles. More recently, it became clear that antigens can also be taken up by dendritic cell extending dendrites in between intestinal epithelial cells (ECs) [5]. As described above for antigens taken up by M cells, those taken up by extending dendrites will ultimately be presented to T cells in MLN for subsequent induction of T and B cell responses. Nasal immunization studies in mice have demonstrated that the NALT serves has the inductive

site for immune responses initiated via the airways. Like the GALT, the NALT is covered by M cells and cervical lymph nodes are believed to play the same role as MLN in the gastrointestinal (GI) tract. The skin is not a strict mucosa. However, it is an epithelium in contact with the external environment. The interest for the skin as a "mucosal" tissue has steadily grown since the observation that transcutaneous immunization can induce immunity in the GI tract [6].

During the last few months and while I was working on this document, the first reports were published indicating that the murine sublingual surface is a distinct and previously unsuspected inductive site for mucosal immunity [7, 8]. As we will see later in this dissertation, our new findings support this notion.

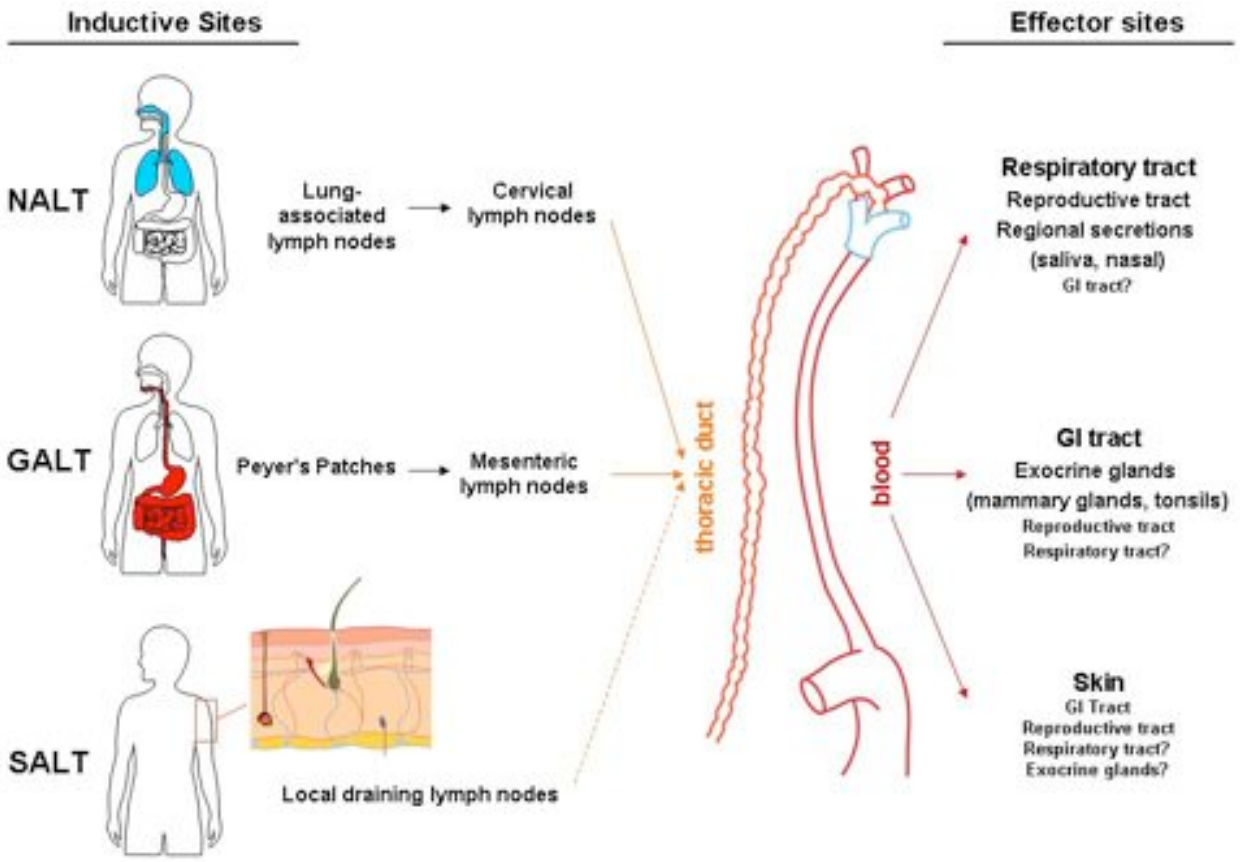

Figure 1. The Mucosa-Associated Lymphoid Tissue (MALT).

The mucosal immune system is divided into inductive sites and effector sites. Inductive sites consist of organized mucosa-associated lymphoid tissue (MALT) as well as local and regional draining lymph nodes, whereas effector sites consist of distinctly different histological compartments, including the lamina propria (LP) of various mucosae, stroma of exocrine glands and surface epithelia. Based on anatomical regions, three most studied subdivisions of the MALT are represented: the NALT or nasal-associated lymphoid tissue, the GALT or gut-associated lymphoid tissue, and the SALT or skin-associated lymphoid tissue. The recently described sublingual site of induction is not depicted. Antigens are sampled at mucosal ifferentiate into memory or effector cells. Not depicted inductive sites and presented to local T and B cells. Sensitized B and T cells leave the site of original encounter of the Ag, transit through the lymph to reach the general circulation and then disseminate between selected mucosal effector sites.

3.1.2.2 Homing to mucosal effector sites

After initial Ag exposure in inductive sites such as the GALT, activated B and T cells home via the thoracic duct and the blood circulation to distant mucosal effector sites, where they differentiate into effector and memory cells to elicit a mucosal immune response (Figure 1).

It is important to note that the homing of effector B and T lymphocytes is governed by specific mucosal homing/tissue receptors, which impose restrictions on their recirculation between mucosal sites. In this regard, $\alpha_4\beta_7$ binds to MAdCAM-1 (mucosal addressin cell adhesion molecule-1), the major homing receptor ligand in the intestinal lamina propria. Chemokines are also directly involved in mucosal homing of effector B and T cells. CCL25 or TECK (thymus-expressed chemokine) is expressed in the small intestine and attracts $CCR9^+$ IgA-commited B cells. Beside CCR9, CCR10 is another chemokine receptor implied in lymphocyte trafficking to the small intestine expressing its ligand CCL28 or MEC (mucosa-associated epithelial chemokine). Mucosal DCs are increasingly recognized as key players in the acquisition of mucosal homing capabilities by effector B and T cells. Thus, retinoic acid produced by GALT DCs (i.e. PP and MLN) appears to be the determinant factor for controlling the expression of integrin $\alpha_4\beta_7$ and CCR9 by B and T cells [9]. As a consequence of the pattern of homing receptors and addressins expressed by cells sensitized in the GI tract, oral immunization induces strong antibody responses in the small intestine, ascending colon and mammary and salivary glands but not in the distal segments of the large intestine, tonsils, and the female genital tract (Figure 2).

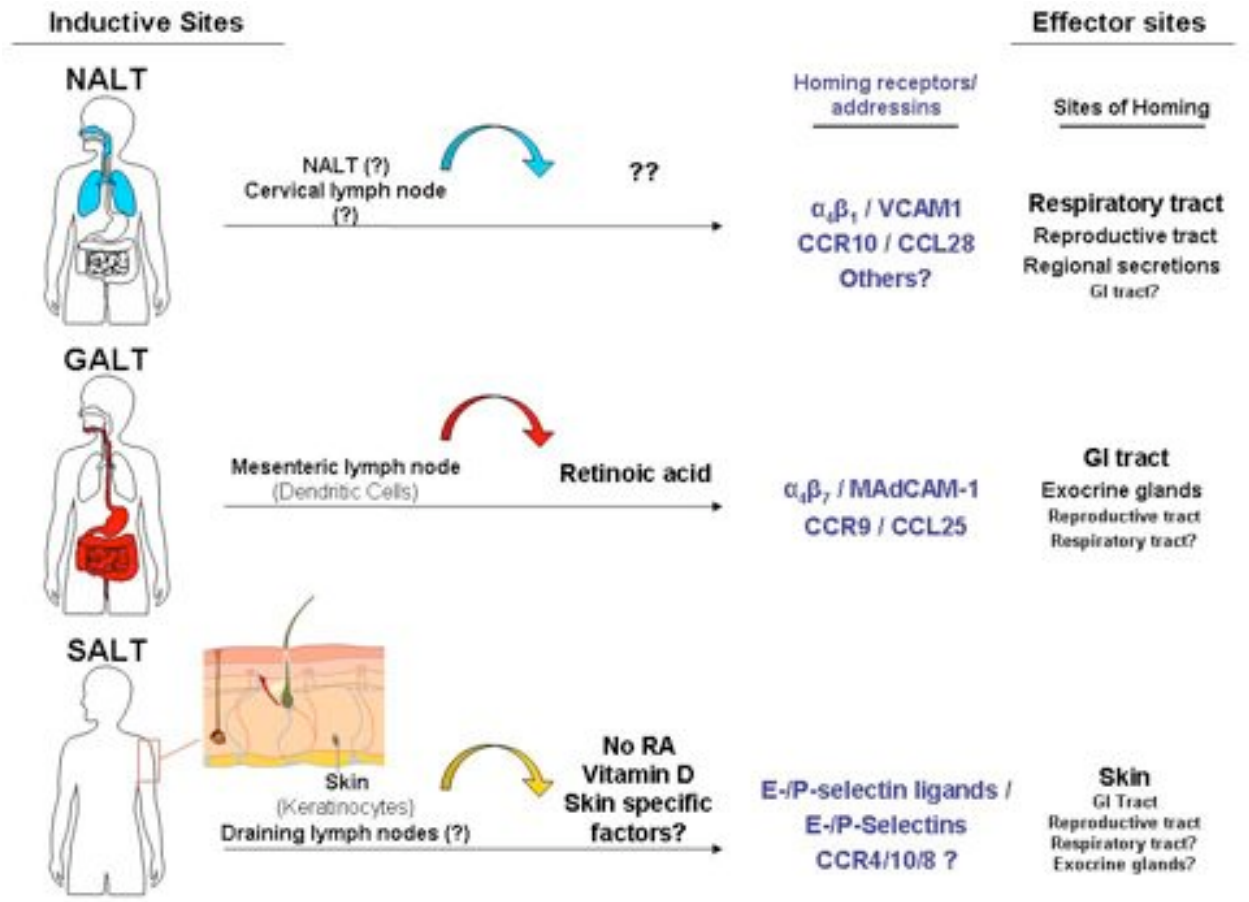

Figure 2. Homing receptors and addressins for mucosal homing of immune effector cells.

The selective homing of cells sensitized in a particular inductive site is determined by site-specific integrins or homing receptors they express on their surface and corresponding mucosal tissue-specific receptors or addressins displayed on vascular tissue. Chemokines produced locally by certain tissues also play a role in attracting cells in inductive sites especially by regulating their surface expression of integrins. Interestingly, factors like retinoic acid produced by mucosal DCs located in the MLN or PP can influence the expression of the integrin $\alpha_4\beta_7$ and the chemokine receptor CCR9 on T and B cells, thereby modulating their migration toward MAdCAM-1 and the gut-associated chemokine TECK/CCL25. Those factors have yet to be identified for inductive sites like the NALT and the SALT. The fact that cytokines, chemokines, integrins are differentially expressed between mucosal tissues explains the compartmentalization of the mucosal immune system which preferentially associates a certain mucosal inductive site with a particular effector site (like the NALT and the respiratory tract, or the GALT and the gastrointestinal (GI) tract).

Rectal and vaginal immunizations give rise to Ab responses essentially restricted to the rectum and the female genital tract mucosa, respectively [10]. One could assume that signals not yet identified, probably provided by local DCs, govern and limit the development of immune responses to these particular sites.

In contrast to the GALT, homing from the NALT or other inductive sites is less well documented. Compelling evidence suggest that $\alpha_4\beta_7$ is not an important homing molecule in the airways, or the urogenital tract. On the other hand, CCR10, $\alpha_4\beta_1$, and L-selectin seem to control the homing from the NALT. For example, activated human tonsillar B cells express CCR10 but

only low to intermediate levels of $\alpha_4\beta_7$, a homing receptor profile that does not favor homing to the small intestinal mucosa. Lack of L-selectin was shown to impair immune responses in the head and neck tissues and the saliva [11]. It has been shown that NALT-derived B cells express CCR10 and $\alpha_4\beta_1$-integrin at high levels, which allows them to migrate to the respiratory and genito-urinary tracts. Interestingly, high levels of antigen-specific IgA and IgG Abs could be induced in cervicovaginal secretions after intranasal immunization in mice, monkeys [12] or humans.

As indicated earlier, interest for the skin as a "mucosal" tissue has steadily grown since the observation that transcutaneous immunization can induce immunity in the GI tract [6]. In this regard, the skin-associated lymphoid tissue (SALT) contains keratinocytes, Langerhans cells, skin trophic T cells, and lymphatic endothelial cells of the skin, but few or no B cells [13]. Lymphocyte homing to the skin is regulated by various homing receptors, including the cutaneous lymphocyte-associated antigen. The cutaneous lymphocyte-associated antigen is a carbohydrate epitope present on memory/effector T cells that infiltrate inflamed skin, where it can interact with both P- and E-selectins. Skin-homing effector T lymphocytes also express chemokine receptors such as CCR4 and/or CCR10, and high levels of $\alpha_4\beta_1$.

The fact that E-selectin is highly expressed on endothelial cells of inflamed skin, female genital tract and oral mucosa suggests that transcutaneous immunization could also promote immune responses in the genital tract [14] and saliva, although this point has not been thoroughly investigated. Retinoic acid, produced by mucosal DCs from the MLN and the PP, favors the expression of gut homing receptors and was shown to block the expression of P- and E-selectin ligands [15]. Vitamin D3 is generated in the skin after sun exposure and converted to the biologically active form $1,25(OH)_2D_3$ through an enzymatic cascade involving 25-hydroxyvitamin D 1 alpha-hydroxylase produced by keratinocytes. In contrast with RA, vitamin D3 suppresses the expression of the gut homing receptors $\alpha_4\beta_7$ and CCR9 and signals T cells to express CCR10 and thus, enables them to respond to CCR10-ligand CCL27 and to migrate to epidermis [16].

The recent publications on sublingual immunization in mice showed broad immune responses with induction of Ab responses in both the GI and respiratory tracts [7, 8]. However, mucosal homing receptors and addressins involved and their regulation remain to be elucidated.

Conclusions from the compartimentalization of the mucosal immune system: The previously described common mucosal immune system is in fact very compartmentalized and this point should be taken in consideration when devising new non-invasive vaccines and immune therapy to be administered via mucosal or cutaneous routes. For instance, immunization via the oral route gives rise to mucosal immune responses that are constraint mainly to the intestinal mucosa, while nasal immunization covers a broader spectrum by inducing mucosal effector cells in the upper respiratory tract, the general circulation and the genitor-urinary tract. In addition, while most basic mechanisms (e.g. lymphoid structures, homing molecules) to enhance secretory immunity are common between mice and humans, some differences may explain inconsistencies between immune responses of mice and humans to mucosal vaccines. Studies in mice have 'set' the nasal route of immunization as an effective method of vaccination to induce immune responses in the systemic and distant mucosal effector compartments; its major asset being to induce optimal and long-term protection in the genital tract, which is not achieved after intra-vaginal immunization or oral immunization in mice. Nevertheless, clinical studies in humans have not shown such consistency in terms of protective mucosal Abs found in the reproductive tract and memory responses. Therefore, new immunization strategies for induction of targeted mucosal immunity are still needed.

3.1.2.3 Polymeric IgA antibodies and mucosal immune responses

Mucosal B cells differ from their systemic counterparts and represent a first line of defense at mucosal surfaces through the active production and exportation of secretory IgA (SIgA) Abs and to a lesser extent secretory IgM (SIgM) Abs.

Polymeric IgA and secretory IgA. The daily production of IgA is greater than any other immunoglobulin class. In contrast with mice, which only have one type of IgA, two subclasses of IgA Abs are present in humans (Figure 3A). IgA1 Abs represent the majority of serum IgA (84 %); they are also present in the tonsils and nasal mucosa. IgA2 Abs predominate in the large intestine and the female genital tract. These IgA Abs consist of a dimer (or a tetramer), linked by a J-chain polypeptide, and covered by a polypeptide chain called the secretory component (SC) [17] (Figure 3A). The SC is derived from the polymeric Ig receptor (pIgR) present on the basolateral membrane of most mucosal epithelia (digestive, respiratory, genital) and glandular epithelia in the mammary, respiratory and lacrimal glands and is responsible for the transport of

polymeric IgA across ECs [18] (Figure 3B). Both IgA subclasses are well exported by pIgR, and give secretory IgA (SIgA). However, IgA2 Abs are more resistant to bacterial degradation than IgA1 Abs [17]. Dimeric IgA secreted by plasma cells in subepithelial tissues binds to pIgR and is internalized by receptor-mediated endocytosis[18]. The complex receptor-IgA is transported into vesicles across the EC to the luminal membrane where the vesicle fuses with the plasma membrane. The pIgR is enzymatically cleaved from the membrane to become the SC, which is bound to and released together with pIgA into the mucous. The same mechanisms apply for the transport of pentameric IgM (Immunoglobulin also containing a J chain) to mucous secretions.

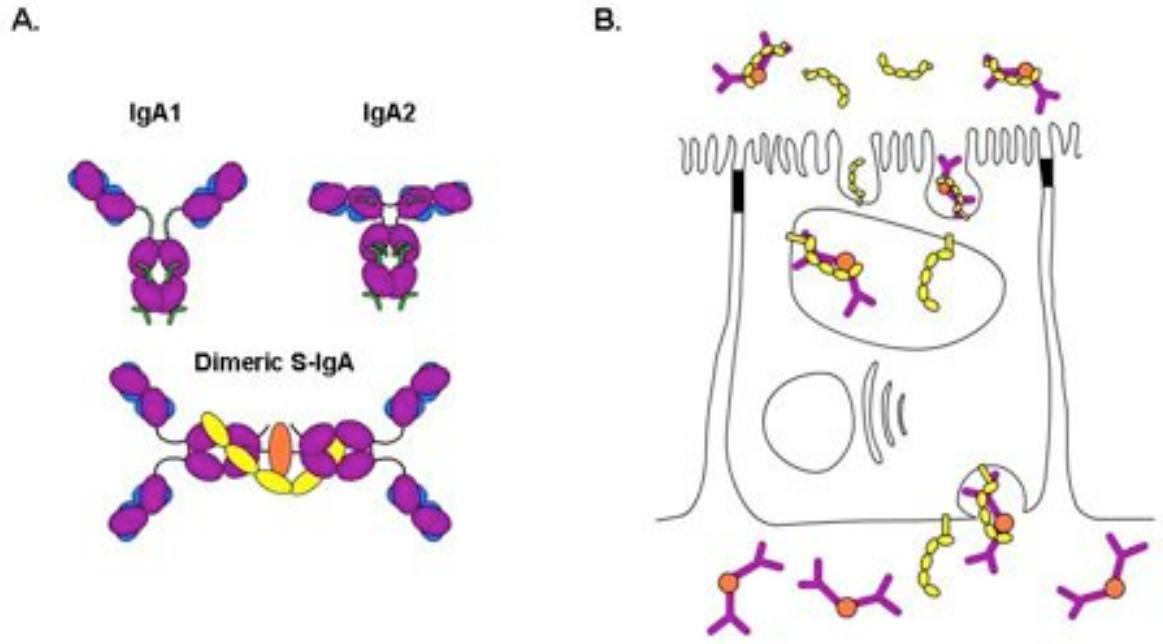

Figure 3. Structure of human IgA Abs and transepithelial transport of secretory IgA Abs.

A. Schematic representations of human immunoglobulins A (IgA). Heavy chains are shown in purple, light chains in blue, J chain in orange, secretory component in yellow. N-linked glycans are shown in green and O-linked glycans by small light blue circles. Top left: IgA1. Top right: IgA2 (Only one allotypic form of IgA2 is represented). Bottom: Dimeric SIgA. B. Schematic representation of the transport of pIgA through a polarized epithelial cell. Polymeric IgA (pIgA) is secreted by plasma cells in the lamina propria at a mucosal membrane. pIgA binds to its receptor, pIgR, on the basolateral surface of an epithelial cell. The complex is endocytosed and transcytosed in endosomes toward the apical surface. There, the pIgR is cleaved, releasing secretory IgA into the lumen. [Adapted from Woof J.M. and J. Mestecky. *Mucosal immunoglobulins*, 2005. Immunol. Rev. 206 (1), 64–82].

Although not considered as a secretory immunoglobulin, IgG is also present in most external secretions at levels comparable to those of IgM. However, in some secretions like the urine, seminal fluid and female genital tract secretions, IgG Abs outnumber SIgA Abs. The IgG Ab can be passively released into mucosal surfaces complexed to IgA or IgM Ab transported across epithelial cells. Other mechanisms independent of pIgR govern the entrance of IgG into

secretions, including passive diffusion. IgE Abs may be carried into mucous membranes and secretions by mast cells and cause their degranulation with local histamine release [19].

Development of SIgA responses. The IgA-secreting B cells are primarily generated through the aid provided by helper T cell to B cell precursors for class-switch from surface (s)IgM to (s)IgA. More precisely, the μ to α isotype switch is mediated by an essential cytokine, TGF-β along with IL-4 and IL-10. The interactions of CD40 on B cells with CD40 ligand on T cells [20], and of ICOS (inducible costimulatory) on activated Th cells with ICOS-L constitutively expressed on B cells [21] are also required for the immunoglobulin switch. This process occurs within the germinal center (GC) of mucosal inductive sites, like the PP. Class-switched B cells expand while homing via the lymph and the blood to the effector sites such as the intestinal lamina propria. There, they continue their differentiation into IgA-producing plasma cells with the aid of IL-5 and IL-6. Because of this dependence on Th2 cytokines, SIgA antibody responses have initially been considered as limited to Th2-inducing stimuli. This notion has been revisited in the last decade, with the observation that SIgA antibodies are also induced by Th1-inducing pathogens/vaccines (i.e., IL-12 [22, 23] and oral *Salmonella* vectors [24, 25]). In addition, Th1 and inflammatory cytokines (IFN-γ and TNF-α) regulate the expression of pIgR on the basolateral membrane of ECs [26] and the expression of SC [27] and thus, the transepithelial transport of polymeric immunoglobulins. Interestingly, it has been shown that the upregulation of expression of pIgR by TNF-α is enhanced by retinoic acid [28].

The T cell-dependent mechanism for induction of IgA Abs can be further enhanced by a crosstalk between intestinal epithelial cells (IEC) and dendritic cells [29]. IEC sampling antigens in the gut lumen release thymic stroma lymphopoietin (TSLP), a signal sensed by gut DCs. In turn, DCs are able to prime and polarize $CD4^+$ Th cells into Th2 cells producing IL-4 and IL-10. IL-6, which is a plasma cell-inducing cytokine, is also a mediator released by these DCs. Thus, together with TGF-β, IL-4 and IL-10 drive mucosal B cells to undergo an IgA-class switch.

T cell-independent (TI) pathways for induction of IgA responses to intestinal flora have been described. In mice, these TI responses involve B1 cells derived from the peritoneal cavity. The mechanism relies on B-lymphocyte stimulator/B-cell-activating factor of the TNF family (BLyS/BAFF) and a proliferation-inducing ligand (APRIL), factors secreted by activated antigen-presenting cells (APCs) (e.g. after exposure to bacteria) and involved in peripheral B cell survival, maturation, and differentiation. Humans lack B1 or B2 cells but a similar mechanism to

mice for the induction of IgA has recently been unveiled. BAFF and APRIL expressed by DCs and mucosal epithelial cells, sensing bacteria in the gut lumen, can trigger a T cell-independent IgA2 class switch in human mucosal B cells [29].

Biological activities of SIgA. More pIgA is transported to the human gut lumen in a day (40 mg/kg body weight, which represents approximately 3 g of SIgA) than the total production of IgG (30 mg/kg). The high avidity due to multiple antigen-binding sites of IgA (and IgM) and the amount of SIgA produced in the gut compensate for their low affinity. SIgA also possess a feature that makes them resistant to proteolytic degradation in harsh environments such as the gut lumen and oral cavity: the secretory component, acquired during the transepithelial transport of dimeric IgA. Furthermore, SC enhances both stability and effector functions of pIgA. As one would expect, SIgA exert functions that differ from those mediated by IgG in the general circulation and that are adapted to the unique environment where these Abs operate [19].

The main function of antibodies secreted in the lumen is to perform immune exclusion of exogenous antigens, by inhibiting uptake of soluble or particulate Ag. By coating commensal as well as pathogenic microorganisms without stopping their growth, SIgA effectively reduce their access to the epithelial surface and at the same time, maintain the balance of the intestinal flora by preventing the outgrowth / invasion of a population over another. Another non-specific mechanism results from glycans present on SIgA and on free SC (Figure 3), which interfere with microbial adherence to mucosal surfaces. Factors present in the mucous can enhance the protective effects of IgA. For instance, the mucin binds SIgA and thus enhances entrapment of SIgA-coated bacteria in the mucus layer. It has become recently appreciated that SIgA adheres selectively to M cells in intestinal Peyer's patches to mediate the transepithelial transport of the Ab molecule and the bound antigen to underlying GALT [30].

Ag-specific functions of SIgA are illustrated by their role in protection against virus infection, such as influenza or human immunodeficiency viruses (HIV). Elegant studies with HIV have shown that pIgA antibodies transiting in epithelial cells during pIgR-mediated transport can neutralize intracellular viruses [31]. SIgA can neutralize various Ag like viruses, by binding to specific epitopes in mucosal secretions. In addition, SIgA can eliminate immune complex formed inside ECs. The role of SIgA in protection against bacteria is more complex. As mentioned above, SIgA do not interfere with commensal or pathogenic bacteria numbers. However, SIgA are necessary for protection of intestinal surfaces against secreted bacterial toxins.

Below the epithelial surface, SIgA display anti-inflammatory properties, which counteract inflammation resulting from complement activation by IgG Abs. However, SIgA can also interact with complement to enhance opsonization of Ag [32].

Summary on secretory antibodies: The SIgM and IgG antibodies can participate in the clearance of mucosal infections, notably in the upper-respiratory and the genital tracts. Nonetheless, SIgA are the major antibodies that contribute to the defense of mucosal surfaces. Serum IgG Abs, can play a major role in the protection of mucosal tissues only in sites where loose epithelial barrier (stratified) allows their leakage from the blood stream. In contrast, SIgA have the unique ability of interfering with the uptake of viruses and macromolecules without requiring or causing tissue damage or alteration of the integrity of the mucosal epithelium. They have been shown to protect against many bacterial toxins and control the initial epithelial colonization of bacterial pathogens, which helps prevent horizontal oral-fecal spread of pathogens and provides herd protections in populations. Thus, it is clear that the induction of specific secretory Abs by a vaccine formulation is a crucial goal for vaccinologists as they could help combat most mucosal infections.

3.1.2.4 Mucosal T cell responses: helper, cytotoxic and regulatory functions

T helper cells in the mucosa. As mentioned above, cytokines secreted by T helper cells play a crucial role in the development of IgA secreting plasma cells. The differentiation of $CD4^+$ T helper cells can be induced in mucosal tissues by APCs that migrate to the interfollicular area of the PP and MLN. It has also been suggested that ECs could process and present antigens themselves to T cells via MHC class I or II molecules and produce cytokines that control the differentiation of Th cell subsets [33]. Like their counterpart in the systemic compartment, two lineages of $CD4^+$ Th cells were described in mucosal tissues based on cytokines produced. Thus, Th1 cells produce IFN-γ and TNF-α, while Th2 cells produce IL-4, IL-6, IL-10 and IL-13 (Figure 4). Recently, it has been shown that the repertoire of $CD4^+$ Th cells is more diverse and that IL-17-producing Th cells or Th17 exist alongside with the previously described Th1 and Th2 lineages (Figure 4). Indeed, studies of autoimmune diseases such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) have highlighted the roles of two cytokines, IL-23 and IL-17, rather than Th1 cytokines as traditionally believed. IL-17 is produced by cells involved in the adaptive branch of the immune system but possesses effector

functions that are characteristic of the innate system. In fact, IL-17 acts like IL-1β, TNF-α, Toll-like receptor (TLR)-4-agonists and as such, represents a link between the innate and adaptive branches of the immune system to induce inflammation. IL-17 also regulates intestinal barrier functions through the induction of tight junction formation [34]. Finally, it has been suggested that TGF-β and IL-6 can induce the differentiation of Th17 cells from naturally occurring $CD4^+$ $CD25^+$ T regulator cell (Treg) precursors [35].

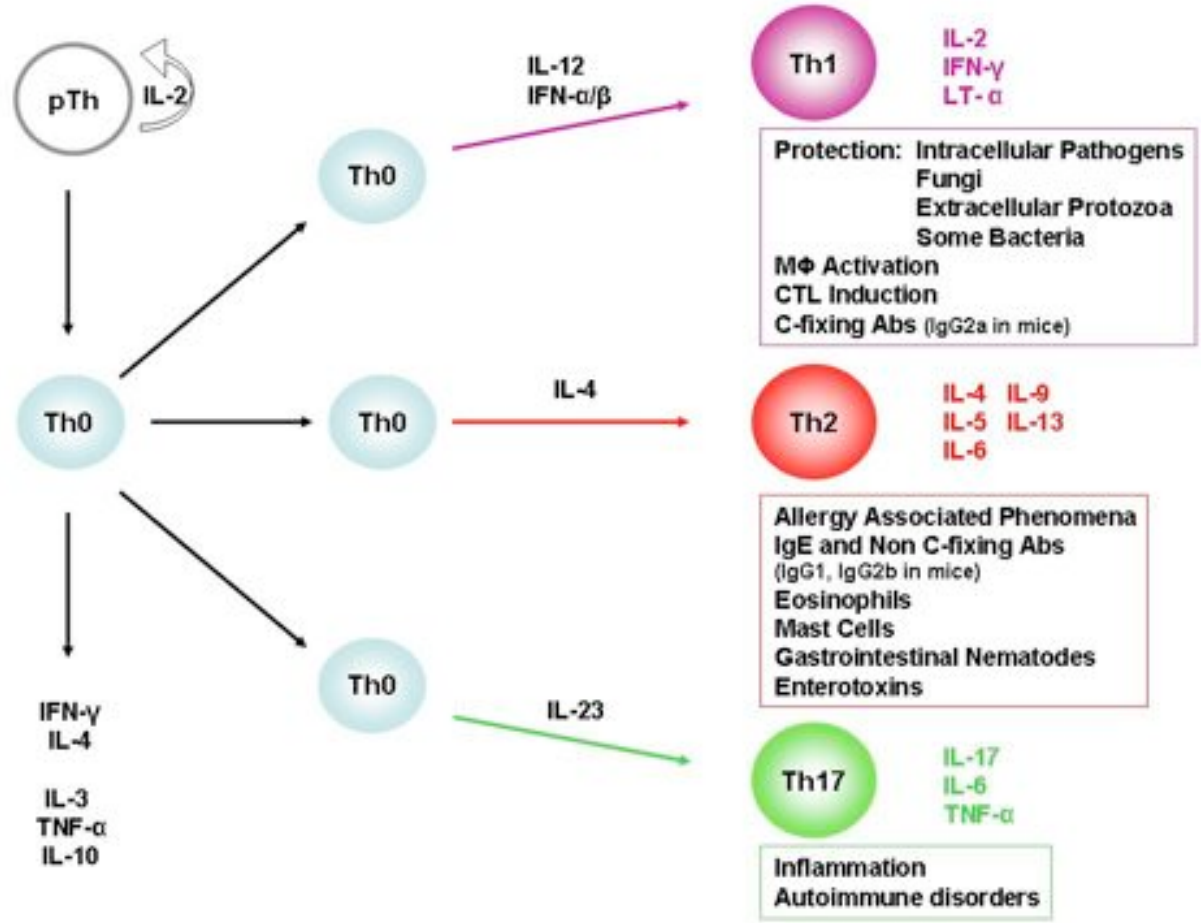

Figure 4. Human and mouse T helper cell subsets.

T helper (Th) cells arise from the thymus. When they acquire CD4, they become precursor Th (pTh) cells. Upon encounter with antigen and stimulation with appropriate cytokines, they start to proliferate. Depending on the nature of the stimulation they can enter the Th1, the Th2 or the Th17 (or IL-17-producing Th cells) pathway. Th1 cells produce IFN-γ, and regulate antigen presentation and cellular immunity. Interleukin-12 (IL-12), which is produced by activated antigen-presenting cells (APCs), drives the process of Th1-cell differentiation. Th2 cells produce IL-4, IL-5 and IL-13, which are important regulators in humoral immunity and allergic responses. IL-4 is required for Th2-cell differentiation. Th17 cells, which require IL-23 for differentiation and produce IL-17, as well as IL-6 and TNF-α, have a dominant role in the development and maintenance of autoimmune inflammation. [Adapted in part from Laurence A. and O'Shea JJ. *T(H)-17 differentiation: of mice and men,* 2007. Nat. Immunol. 8: 903].

Mucosal cytotoxic T lymphocytes (CTLs). Cytotoxic functions of mucosal T cells represent a crucial backup mechanism for mucosal protection in case of a defective or insufficient IgA Ab

response development against a viral or a bacterial infection. A majority of mucosal CTLs are $CD8^+\alpha\beta$ T cells and are mostly found at effector sites, such as the intestinal lamina propia. Just like IgA-producing plasma cells, mucosal CTLs derive from precursors in inductive sites (i.e., PP and MLN). CTLs differentiate with the aid IL-2 produced by Th1 cells into effector CTLs and their maintenance and survival is dependent on IL-7 and IL-15. Mucosal CTLs recognize antigen on MHC class I molecule and are then able to kill their target or a virus-infected cell through the secretion of perforin, a protein that forms pores in infected cell membranes, and cytolytic granules full of granzyme proteases. It has been established that CTLs can be induced in systemic and mucosal tissues after oral [36], nasal [37], rectal [38], vaginal and after transcutaneous immunizations [6]. For example, oral immunization with a live recombinant *Mycobacterium bovis* BCG vectors expressing SIV nef gives rise to the development of antigen-specific CTL responses in inductive sites (PP and MLN), in effector tissues (intestinal LP), as well as in systemic lymphoid tissues [39]. Studies with knockout mice have demonstrated the requirement of $CD4^+$ Th cells for induction of CTLs; however, cytotoxic T-cell responses were also reported *in vivo* in the absence of CD4 helper T cells [40].

Regulatory T cells. Most of the time, the mucosal immune system ignores external microorganisms (i.e., commensal bacteria) and antigens (i.e., food antigens) and does not mount an immune response against them. Mucosal homeostasis can also be maintained by specifically suppressing a cellular and/or a humoral immune response, which would be detrimental for the integrity of the mucosal surfaces. Mucosal tolerance concerns mainly the T cell compartment as delayed-type hypersensitivity (DTH) and *in vitro* T cell proliferation responses are diminished [41].Various active mechanisms are involved in mucosal tolerance, including activation-induced cell death, deletion and/or anergy of Ag-specific T cells, induction of regulatory T cells and production of suppressive cytokines [41].

To date, four types of regulatory T cells were described: 1) the antigen-specific $CD4^+$ Th2-like cells that produce IL-4 and IL-10 and antagonize the activity of Th1 effector cells [42]; 2) the $CD4^+CD45RB^{low}$ Tr1 cells producing IL-10 [43]; 3) the TGF-β-producing Th3 cells [44]; 4) and $CD4^+CD25^+$ regulatory T cells or Treg cells [45, 46]. Tregs are naturally occurring regulatory T cells; they are $CD4^+$ and express the IL-2-receptor α chain (CD25). Because they are anergic, they do not proliferate after allogeneic or polyclonal stimulation and produce small amounts of cytokines. Once activated, they block proliferation of and cytokine production in $CD4^+$ or $CD8^+$

T cells in a cell contact-dependent manner. Notably, Treg cells immediately confer suppressive properties to $CD4^+$ T cells, a process that has been termed "infectious tolerance". In turn, these induced-Th suppressor cells display suppressive activities that are cell contact-independent (cytokine-mediated).

Recent studies have provided a link between Tr1 cells, Th3 cells and Tregs. Indeed, it has been shown that there are two subsets of Tregs with different properties. This dichotomy is based on their expression of one of the two integrins, $\alpha_4\beta_7$ and $\alpha_4\beta_1$, one being a homing receptor to mucosal site, the other to inflamed tissues, respectively. Studies have demonstrated that co-culture of $\alpha_4\beta_1^+$ Tregs with conventional $CD4^+$ T cells induce Th3-like TGF-β secreting cells while $\alpha_4\beta_7^+$ Tregs in the same culture conditions induce IL10-producing Tr1 like cells and the induction of Foxp3, a transcription factor thought to be expressed solely on Tregs [47].

While the induction of mucosal tolerance may be useful to treat allergies or autoimmune diseases, the development of a regulatory mechanism in response to a mucosal vaccine against an infectious disease is certainly not desirable. Therefore, appropriate vaccine formulations with optimal antigen / adjuvant / delivery system combinations are needed to overcome suppressive responses and elicit strong immune responses.

Summary on mucosal T cells and relevance for vaccine strategy: Regulatory T cells prevent the host from developing excessive reactions the myriade of microorganisms presnt in mucosal surfaces. Cytotoxic T cells can also provide mucosal tissues with specific and non specific protection of against pathogens. These responses as well as the magnitude and quality of Ab responses are orchestrated by T helper cells. Therefore, a major challenge for vaccinologists is to induce the T helper subset(s) that will more effectively support the needed immune responses for optimal protection. In this regard, the following parameters have important implications on the nature of T helper cell subset responses: (1) the adjuvant, (2) the vaccine carrier, (3) the presence of regulatory cytokines, (4) the route of administration, and (5) the quantity and nature of the antigen.

3.2 Mucosal vaccines and adjuvants

3.2.1 Mucosal vaccines against infections

As mentioned earlier, the most common sites of entry for infectious agents are mucosal surfaces. Because only immune responses initiated in mucosal tissues give rise to effector cells with mucosal homing capabilities, local protection of these sites can only be achieved by mucosal vaccination. Other major advantages of mucosal vaccinations are the following: 1) The administration is painless and does not require trained medical personnel; 2) These needle-free procedures eliminate the risk of cross-infections by blood-borne pathogen though contaminated needles.

The development of mucosal adjuvants has long been empirical, with variable degree of success. It has now become clear that the design of effective mucosal vaccines and delivery systems should take into account specific hurdles associated with the mucosal route considered. For example, intact proteins are usually destroyed in the GI tract or their uptake is limited, proteolysis of vaccine antigen by enzymes, and interferences with local antigens and flora are the main obstacles in the case of oral delivery. Regardless of the route of immunization, it is important to confer the adequate immunostimulating properties to the mucosal vaccine for the induction of adaptive immunity. This is usually the role of the adjuvant. To date, several mucosal vaccines are either currently used or under development for mass vaccination.

3.2.1.1 Oral polio vaccine

The live-attenuated orally administered polio vaccine (Sabin OPV), introduced in 1961, is the first known oral vaccine. The OPV was superior at inducing mucosal Ab responses than the parenteral inactivated polio vaccine (Salk IPV) released in 1955. An enhanced formulation of the parenteral vaccine (e-IPV) was later found to induce some IgA Abs in nasal secretions, although to a lower extent than after OPV or infection with wild-type virus. This observation raised the debate that some mucosal immunity could be induced by parenteral immunization. However, this information is contentious because data was collected among populations who could also receive OPV or who were located in poliovirus endemic areas. Therefore, it is still unclear whether parental e-IPV can induce mucosal immunity. However, it is unambiguous that individuals that have been either primed by OPV or naturally exposed to the live poliovirus can develop a strong memory mucosal IgA response [48]. The OPV remains the vaccine of choice for use against

epidemic outbreaks of poliomyelitis due to its ability to rapidly induce development of intestinal immunity, and to provide indirect vaccination for those coming into contact with immunized patients. Nonetheless, better attenuation of the vaccine strain would be needed to prevent potential reversion to wild-type and subsequent risk of vaccine-associated paralytic poliomyelitis [49, 50].

3.2.1.2 Vaccines against enteric infections

The World Health Organization (WHO) estimates that diarrheal diseases account for 15-34 % of all deaths in certain countries and cause 4 to 6 million deaths a year, mostly children, eldery and immunocompromised people. While efficacious vaccines exist against *V. cholerae*, *S. typhi* and rotavirus, none are available against enterotoxigenic *E. coli* (ETEC) and *Shigella* (Table 1).

Cholera vaccines. Cholera is one of the best-studied mucosal infections. Until recently, the only vaccine available consisting of phenol-killed whole cells of *Vibrio cholerae*, was administered parenterally in two doses at two-week interval and reached a 50% protection in individuals that lasted no longer than 6 months. Presently, two oral vaccines are available. Dukoral is made of killed whole cells *Vibrio cholerae* O1 combined with recombinant binding subunit CTb. It has proven to be safe and efficient in several trials. The live attenuated vaccine CVD 103-HgR, containing a genetically manipulated classical *V. cholerae* O1 Inaba strain, with a deletion in the gene encoding the enzymatic subunit cholera toxin A, is the second internationally licensed oral cholera vaccine. While some studies have shown very high levels of protection (>90%), a large field trial in Indonesia yielded only 24% protection in the first year [51].

Typhoid fever vaccines. Typhoid fever is caused by *Salmonella enterica*, serovar typhi, commonly called *Salmonella typhi*. It is responsible for 600,000 deaths a year according to the WHO. The parenteral heat phenol inactivated whole cell vaccine is still in use in some developing countries but is associated with fever and systemic reactions. There are now two other vaccines available against typhoid fever, both very well tolerated. An orally administered, live-attenuated vaccine made from the Ty21a strain of *Salmonella typhi*, and a Vi capsular polysaccharide vaccine (ViCPS) (Sanofi Pasteur) for intramuscular use in one dose. None provide total protection but both reduce typhoid fever. Presently, efforts are being made to search more immunogenic strains for oral vaccines [52].

Rotavirus vaccines. Rotaviruses are the most common cause of severe diarrhea in children. It is responsible of about 600,000 deaths annually worldwide. Two virus surface proteins, VP4 and

VP7, are targets of neutralizing antibodies and either antibody can mediate protection. In 1998, the highly efficacious vaccine Rotashield, a live, orally administrated, tetravalent human-simian reassortant (RRV-TV) vaccine containing four serotypically distinct VP7 components, was licensed but withdrawn shortly after because of elevated cases of intussusception (i.e. intestinal invagination resulting in obstruction). In 2006, two new vaccines were introduced. Rotarix is a monovalent live-attenuated human rotavirus vaccine (made of a tissue-culture-adapted human P1A[53]G1, VP6 subgroup II and NSP4 geno-group B strain), administered in two doses orally in infants of 2 and 4 months of age. RotaTeq is a pentavalent bovine-human reassortant vaccine. It contains several pieces of nucleic acid from human and bovine parent rotavirus strains; it is composed of the five strains, each containing a human rotavirus gene encoding the VP7 neutralizing protein from different serotypes. This vaccine is given orally in a three dose-schedule in infants who are 2, 4 and 6 months old. Rotarix and RotaTeq are considered equivalent in their protective effects [54, 55].

3.2.1.3 Vaccines against respiratory infections

Influenza virus. A trivalent live attenuated cold-adapted vaccine FluMist has been developed for intranasal spray delivery and licensed in the USA for vaccination of persons from 5–49 years of age [56]. The vaccine is safe, effective, and shows remarkable genetic stability, but has to be kept at -18°C. When compared with injectable influenza vaccine, live attenuated influenza vaccine has demonstrated broader serum antibody responses, particularly against antigenically mismatched influenza A strains [57]. A new, heat-stable version of the vaccine has recently been developed, and has shown remarkable efficacy in clinical trials. Other influenza vaccines, currently being developed, include a synthetic peptide influenza vaccine for nasal administration. In an attempt to elicit a higher antibody response, PowderJect (USA) developed a needle-free delivery method to administer influenza vaccine called epidermal powder immunization and shown elevated humoral immune responses in humans [58]. Virosomes are small (150 nm) spherical vesicles composed of a lipid membrane with integrated envelope proteins derived from influenza virus, predominantly hemagglutinin. They differ from virus-like particles in that they are semisynthetic particles reconstituted *in vitro* from lipids and from virus-derived proteins. An intranasal virosomal subunit influenza vaccine with heat labile toxin from *Escherichia coli* (LT-I) as the adjuvant elicited strong systemic and mucosal Ab responses in healthy adults, with the presence of neutralizing secretory IgA Abs in nasal secretions [59].

Respiratory Syncytial Virus (RSV). The RSV causes lower respiratory tract infection in infants and reinfections can occur in all age groups. To date, there is no licensed vaccine against RSV. Most attenuated live RSV strains tested in humans caused mild to moderate congestion in the upper respiratory tract of infants of one to two months old and, therefore, were considered as insufficiently attenuated for early infancy. Intranasally delivered, genetically engineered attenuated or vectored vaccines are currently most promising for newborns. Maternal vaccination may be the optimal strategy to protect the very young infants [60].

3.2.1.4 Vaccines against genitourinary infection

Human Papillomavirus (HPV) serotypes 16 and 18 are associated with the development of cervical cancer [53, 61]. HPV is a nonenveloped, encapsulated, double-stranded DNA virus. The HPV L1 capsid protein can generate virus-like particles (VLP) when expressed in heterologous system such as yeast. VLPs are non-infectious and immunogenic without adjuvant, can induce neutralizing antibodies and be protective against viral challenge. A tetravalent vaccine composed by virus-like particles (VLPs) of HPV 16, HPV 18, HPV 6 and HPV 11 has been approved in the USA [62]. The VLPs are manufactured in *Saccharomyces cerevisiae* and administered with aluminum adjuvant in three intramuscular injections. Research efforts are now focusing on an alternative route of administration and on the induction of immunity in the genital mucosa to overcome the variation of Ag-specific antibodies in the cervix seen in ovulating women. Nasal administration of HPV16 VLPs along with LT-I in mice has been shown to elicit strong systemic and mucosal Ab responses, with the presence of SIgA in vaginal secretions, as well as cell-mediated immunity against VLPs [63].

Mucosal infection	Vaccine	Route of administration
Polio	Live attenuated vaccine (OPV)	Oral
Enteric infections		
Cholera	- CVD 103-HgR live, attenuated *Vibrio cholerae* strain - Killed whole cells *V. cholerae* O1 combined with recombinant CTb subunit	Oral
Typhoid fever	Purified capsular polysaccharide Vi antigen	Subcutaneously or intramuscularly
Rotavirus	- Monovalent rotavirus vaccine - Pentavalent bovine-human reassortant	Oral
Shigella	None	
ETEC	None	
Respiratory infections		
Influenza virus	Trivalent live attenuated cold-adapted influenza virus reassortant strains	Nasal
Respiratory syncytial virus	None	
Genitourinary infections		
Human papilloma virus (HPV)	Tetravalent vaccine: virus-like particles (VLPs) of HPV 16, HPV 18, HPV 6 and HPV 11	Intramuscular

Table 1. Mucosal vaccines against infections.
[Adapted from Holmgren J. and C. Czerkinsky. *Mucosal immunity and vaccines*, 2005. Nat. Med. 11, S45 - S53].

3.2.2 Vaccine adjuvants and mucosal adjuvants

3.2.2.1 Definition and characteristics of an adjuvant

The word adjuvant comes the Latin word *adjuvare*, which means to help, to assist. Adjuvants are a group of heterogeneous substances that enhance or modulate the immunogenicity of a co-administered antigen. Subunit vaccines are generally considered as a safer alternative to vaccines consisting of whole cell/bacteria or live virus. However, these vaccines tend to promote weak immune responses. Adjuvants are being used to: 1) enhance the immunogenicity of highly purified or recombinant antigens; 2) reduce the amount of antigen or the number of immunizations needed for protective immunity; 3) improve the efficacy of vaccines in newborns, the elderly or immuno-compromised persons; or 4) as antigen delivery systems, for the uptake of antigens by the mucosa [64]. Mucosal adjuvants and delivery systems are indeed required for efficient antigen delivery to mucosal surface, preventing the vaccine from degradation and enzymatic digestion, especially at the intestinal surface. As will be reviewed below, mucosal adjuvants can guide vaccine antigens toward mucosal inductive sites and stimulate their uptake

by resident APCs or allow their passage through mucosal the epithelial barrier. Mucosal adjuvants activate the development and control the polarization (i.e., Th1 or Th2) of adaptive immunity for effective protection at various mucosal effector sites, as well as in the systemic compartment. Several molecules were described as mucosal adjuvants. Their mechanisms of action depend upon their structures and the nature of interactions with immune cells. The best described mechanisms include stimulation of APCs with specific ligands (Toll-like receptors or TLRs, and other innate receptors), cytokine and/or chemokine induction, complement activation, recruitment of different cell populations, delivery to different APC, regulation of the expression of MHC class I or class II and costimulatory molecules and stimulation of the production of different subtypes of antibodies.

3.2.2.2 Alum, the gold standard adjuvant

Aluminium salts (aluminum hydroxide, aluminum phosphate, potassium aluminum sulfate or "alum") are the only adjuvants allowed for use in human vaccines. However, they have weak or no adjuvant effect with certain antigens. They are unable to induce cell-mediated and cytotoxic T cell responses and are associated with Th2 and IgE responses. Finally, they display no adjuvant effect when delivered via the oral or nasal route [65].

3.2.2.3 Toll-like receptors and their importance in the mode action of adjuvants

Mammals are equipped with both innate and adaptive immune systems to fight against infections. As a first line of defense, the pattern-recognition receptors (PRRs) on innate immune cells (macrophages, epithelial cells…) are able to sense the microbe- or pathogen-associated molecular patterns (MAMPS or PAMPs) present in microorganisms [66]. A variety of antimicrobial responses ensue to combat infection. Meanwhile and if the innate immune system is overwhelmed, the adaptive branch of the immune system generates very specific and powerful immune responses, with the development of Ag-specific T and B lymphocyte and memory responses. Functionally and not only chronogically, the innate immunity has a crucial role in that it shapes the following adaptive immune response, particularly through Toll-like receptors (TLRs) and other PRRs.

TLRs can be expressed at the surface (e.g. TLR2 and 4) or located in endosomes (e.g. TLR3 and 9). TLRs are distributed differentially between APCs subtypes (e.g. myeloid, plasmacytoid, epidermal DCs…). Well-characterized MAMPs or TLR-ligands include lipopolysaccharide

(LPS, Gram negative bacteria), peptidoglycan (PGN, predominantly Gram positive bacteria), lipoteichoic acids (LTA), lipoproteins (e.g., muramyl dipeptides), flagellin, bacterial cell-wall polysaccharide structures (e.g., mannoses), dsRNA (produced during viral infection), and unmethylated CpG DNA (found in most bacterial species and some DNA viruses). Interestingly, most of can also act as adjuvants [67].

Indeed, APCs can be activated through TLRs sensing of specific bacterial, viral, fungal or protozoan signals. Then, activated APCs can modulate the adaptive immune system by orientating Th responses toward a Th1- or Th2-cell response. For instance, LPS agonists, imidazoquinolines and unmethylated CpG oligonucleotides induce Th1 responses after sensing by TLR4, 7/8 and 9, respectively. TLRs play a role in generating T-cell receptor (TCR)-ligands on APCs, which help present preferentially microbial antigens (over self-Ag) with MHC class II molecules to T cells [68]. TLRs not only trigger inflammatory responses, but also regulates them through Treg cells expansion, to help clean after a resolved infection [69, 70].

Because TLRs are crucial instructors in the development of adaptive immune responses, their mechanisms of action are being dissected and various combinations, routes of administration are being tested, in order to design adjuvants that can finely modulate immune responses without causing detrimental effects to the host.

3.2.3 Current mucosal adjuvants

As suggested above, many current adjuvants act completely or partially via TLR and are usually isolated from or analogs of bacterial compounds (CpG, CT…); or they trigger (CT, LT…) or are TLR-downstream mediators (cytokines, chemokines). Others function like delivery vehicles (chitosan nanoparticles).

3.2.3.1 Enterotoxins and non-toxic derivatives

The enterobacteria *Vibrio cholerae* and *Escherichia coli* produce two main toxins, cholera toxin (CT) and heat labile toxin (LT), respectively. These enterotoxins have an AB5-type structure. The binding subunit (CTb or LTb) is a homopentamer, responsible for the binding to intestinal epithelial cells when ingested. CT and LT-I bind with high affinity to GM1-gangliosides, receptors ubiquitously present. In addition, LT-I also binds polyglycosylceramides, asialo-GM1, GM2 and polylactosamine-containing glycoproteins. The A subunit is composed of a globular

A1 domain with an ADP-ribosyl transferase activity and an A2 domain that interacts with the B subunit. After binding of the B subunit to the cell surface, and cleavage of the A2 fragment, the A1 fragment enters the cytosol, enzymatically ribosylates membrane-bound G protein $G_s\alpha$ that, in turn, stimulates an adjacent adenylate cyclase, leading to an increased cAMP production (Figure 5).

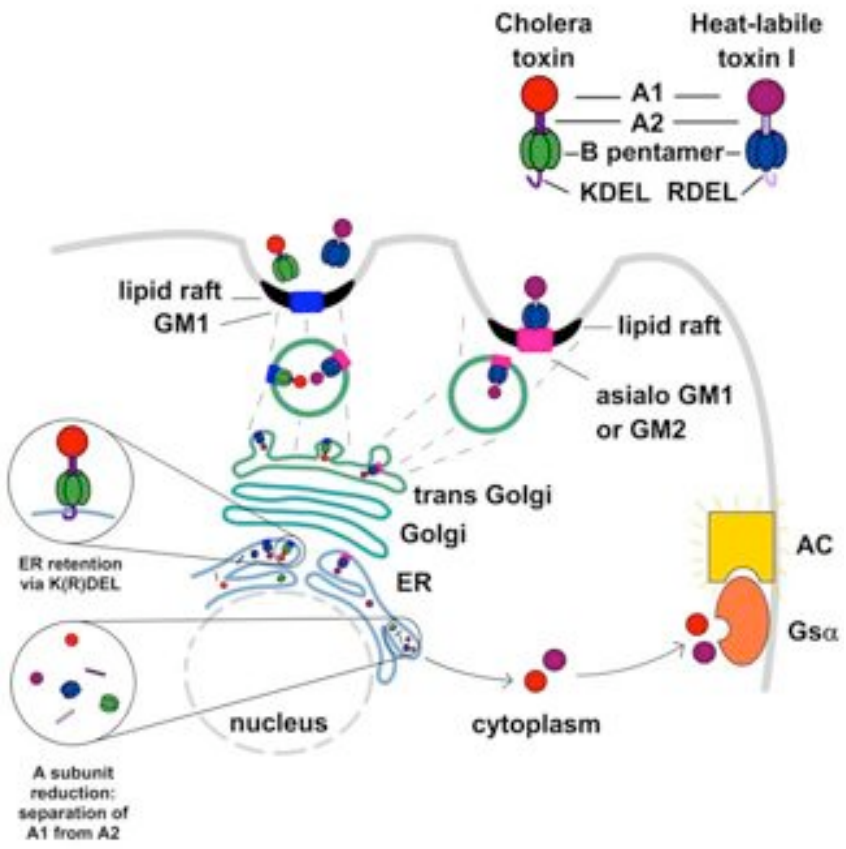

Figure 5. Enterotoxins CT and LT-I cell entry.

CT and LT-I holotoxins bind to membrane GM1 gangliosides. Additionally, LT-I can bind asialo-GM1 or GM2 receptors. After endocytosis, the CT- and LT-I-receptor complexes traffic retrograde through Golgi apparatus into the lumen of the endoplasmic reticulum (ER). The A2 subunit of CT contains an ER retention signal tetrapeptide KDEL (RDEL in LT), which plays a role in the movement of CT (LT) from the Golgi apparatus to the ER. Once there, the A1 peptide is unfolded and dissociated from the B pentamer. The B subunit seems to remain associated to the ER. The A1 subunit is released into the cytosol, reaches and ADP-ribosylates the heterotrimeric GTPase Gsα on the cytoplasmic surface of the basolateral membrane, which leads to the activation of an adenylate cyclase and thus production of cAMP. [Adapted from Chinnapen D.J.-F., et al., *Rafting with cholera toxin: endocytosis and trafficking from plasma membrane to ER*. FEMS Microbiol. Lett., 2007. 266 (2), 129–137].

The enterotoxins CT and LT-I are powerful mucosal adjuvants. Their adjuvant properties cover a broad spectrum of adjuvant functions described earlier. By binding to cell surface gangliosides, the B subunit of these enterotoxins can target vaccines to mucosal ECs. CT and LT-I can also enhance immune responses by increasing the permeability of epithelial membrane [71]. CT plays a role in B cell switch and subsequent production of SIgA Abs. CT and LT-I also play a role in modulating $CD4^+$ Th responses. When delivered through the GI or respiratory tract, the adjuvant activity of CT is associated with IL-4 and Th2 responses [72, 73, 74] while LT-I triggers mixed

CD4$^+$ Th1 and Th2 responses [75]. The development of mutant derivatives / chimeras of CT and LT-I with low or no enzymatic activity has shown that the ADP-ribosyltransferase activity was a necessary requirement for both the adjuvant activity of these molecules and the pattern of Th cell responses they promote [76, 77]. Both CT and LT-I upregulate costimulatory and MHC class II molecules expression by macrophages and dendritic cells and help the interaction of these activated APCs with effector B and T cells [78, 79, 80]. It has been shown that CT induces IL-1 secretion by APCs, a cytokine involved in mucosal immunity (see Cytokines section for detail on IL-1 as mucosal adjuvant) [81].

CT and LT-I possess all the characteristics to be ideal mucosal adjuvants; however, their high toxicity, which is intimately linked to their adjuvant activities, prevents their use in humans. Toxicity is. The watery-type diarrhea induced by these enterotoxins is the most striking illustration of their toxicity via the oral route. It is caused by the increase in cAMP induced by the ADP-ribosylating subunit A inside intestinal epithelial cells, which results in the secretion of water and chloride ions into the lumen of the small intestine. Due to the abundance of GM1 gangliosides including in brain tissues, GM1-binding CT or LT-I given nasally, can target the central nervous system tissues by a mechanism of retrograde transport and are associated with an inflammatory response (Figure 6).

	Cholera toxin (CT)	Heat labile toxin I (LT-I)
Increase cAMP	+++ ADP ribosylation	+++ ADP ribosylation
GM1-receptors	GM1	GM1,asialo GM1
Adjuvanticity		
T helper	Th2 >> Th1	Th2 and Th1
S-IgA	+++	+++
Target CNS	Yes	Yes

Figure 6. GM1-binding enterotoxin adjuvants CT and LT-I.

Cholera toxin (CT) produced by *Vibrio cholerae* and heat-labile toxin I (LT-I) produced by *Escherichia coli* consist of pentameric binding subunit B, an A1 subunit baring the ADP-ribosyl activity and an A2 subunit, linker between A1 and B subunits. Both bind GM1 gangliosides and LT-I also binds other gangliosides. This differential pattern of

binding receptors is believed to lead to the difference in their Th responses. Both are potent mucosal adjuvants capable of inducing secretory IgA Abs. Unfortunatley, they also target the central nervous system tissue after nasal administration.

Another serogroup of enterotoxins include the LT-II of *E. coli* [82], which shares the inability to bind to GM1-gangliosides [83]. Two antigenic variants of LT-II, designated LT-IIa and LT-IIb, with different binding specificities for ganglioside receptors have been isolated [82, 84, 85]. The B subunit of LT-IIa binds with high affinity to GD1b and less strongly to GM1, GT1b, GQ1b, GD2, GD1a, and GM2 [83]. Unlike CT and LT-IIa, the LT-IIb lacks affinity for GM1 but has been shown to bind with high affinity to the disialoganglioside GD1a [83]. Both LT-IIa and LT-IIb were shown too be nasal adjuvants and enhanced systemic Ab responses via mechanisms involving mixed Th1 and Th2 responses with both antigen-specific IL-4- and IFN-γ-producing cells [86]. Other studies showed that, unlike GM1-binding enterotoxins, LT-II enterotoxins were inactive in stimulating IL-1 and IL-8 secretion by APCs [87]. 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 [87] (Table 2).

A CTA1-DD molecule was engineered by linking the enzymatically active A subunit domain of the toxin to a dimer of an Ig-binding fragment, D, of *Staphylococcus aureus* protein A [88]. CTA1-DD targets the ADP-ribosyltransferase towards B cells only and thus circumvent GM1-receptor and potential retrograde transport into the CNS after nasal administration (Table 2). In order to generate immune responses via the oral route, Mowat et al. successfully incorporated CTA1-DD and an antigen in lipophilic immune stimulating complexes (ISCOM) containing the saponin derivative QuilA without observing any toxicity [89].

One of the most significant recent development regarding the use of enterotoxins as vaccine adjuvants is probably the finding almost a decade ago that CT can act as adjuvant for vaccines applied on the skin and other epithelium to promote antibody responses in mucosal secretions. In this regard, CT applied on the skin along with F1-V antigen from *Yersinia pestis* could confer protection against challenge by injection of a virulent strain of plague [90]. More recently, immunization through the sublingual epithelium was shown to be comparable to intranasal immunization and superior to oral immunization regarding the magnitude and anatomic dissemination of the induced immune responses [7].

Bacterial Products or Derivatives	Molecular targets	Known target cells	Immune responses promoted	
			Systemic	Mucosal
LT-IIa	GD1b>>GT1b,CG1b,GD2, GD1a, GM2, GM1	Macrophages (MØ) Dendritic cells (DC), T cells	Yes	Yes
LT-IIb	GD1a	MΦ, DC, T cells	Yes	Yes
CT-A	Unkown	Unkown	Yes /(?)[b]	Yes / (?)[b]
LT-A	Unkown	Unkown	Yes / (?)[b]	Yes / (?)[b]
CTA1-DD	Surface immunoglobulins	B cells	Yes	Yes

[a] Most studies examined the adjuvant activity for nasal vaccines.
[b] (?) Denotes controversial findings due to lack of understanding of mechanisms involved and/or only partial information about potential contaminants.

Table 2. Non-GMI-targeting bacterial toxins and derivatives tested as adjuvant for mucosal vaccines.

[From Duverger et al. Non-GM1 ganglioside-targeting bacterial toxins and derivatives with mucosal adjuvant activity, 2005. Int J Oral-Med Sci. 4: 53].

3.2.3.2 Zonula occludens toxin

In 1991, Fasano et al. identified a toxin in some CT-culture supernatants of *Vibrio cholerae* strains that could increase the permeability of small intestine mucosa by reversibly affecting the structure of intercellular tight junctions or zonula occludens and thus named it Zonula occludens toxin or Zot [91]. Nasal immunization of mice with ovalbumin, a soluble Ag, and recombinant Zot, either alone of fused to the maltose-binding protein (MBP-Zot) induced high OVA-specific IgA Ab titers in plasma and in mucosal secretions. Zot also induced Ag-specific IgG subclass responses that resembled the pattern induced by LT-I [92]. In addition, Zot acts as an adjuvant for rectal immunization with tetanus toxoid, with elevated titers in anti-TT IgG Abs after the third immunization. Mucosal responses were however not analyzed [93]. Zot displays low immunogenicity compared with the adjuvant LT-I.

3.2.3.3 Chitosan

Chitosan is a cationic polysaccharide, consisting of repeating units of N-acetyl-D-glucosamine and D-glucosamine, derived from the partial deacetylation of chitin, which is commercially prepared from crustacean exoskeleton. Like Zot, soluble chitosan formulations can open the tight junctions and enhance paracellular transport across the epithelium of both nasally and orally co-administered macromolecular drugs [94, 95, 96, 97, 98, 99]. Most propably, chitosan acts by

opening transiently the tight junctions between epithelial cells. Thus, like Zot, chitosan enhances the penetration of antigens across epithelia. When administered nasally with a mixture of *Bordetella pertussis* filamentous haemagglutinin (FHA) and recombinant pertussis toxin (rPT), chitosan increased serum IgG Abs and secretory IgA Abs in lung lavage and nasal washes to both antigens [100]. Additionally, chitosan can be used to prepare microparticles or nanoparticles to develop particulate vaccine delivery systems that are taken up by the M cells and subsequently biodegraded. Chitosan nanoparticles can easily be generated and loaded with antigen for uptake by intestinal Peyer's patches [101]. Nanoparticles composed of chitosan complexed with DNA coding for a dominant peanut allergen gene were orally administered and resulted in elevated SIgA Abs as well as serum IgG2a [102]. The safety of chitosan has also been successfully addressed in human trials [103, 104], however, induction of protective immunity could require co-administration of other adjuvants such as LTK63, a non-toxic mutant of LT-I [105].

3.2.3.4 Immunostimulatory DNA sequences

Bacterial DNA or synthetic oligodeoxynucleotides containing unmethylated Cytidine-phosphate-Guanosine motifs (CpG ODN) act via TLR9, a receptor present on DC, B cells, MØ. CpG motifs can induce B cell proliferation and immunoglobulin synthesis. CpG motifs create a Th1-environment in trigerring the secretion of Th1-type cytokines in various immune cells (IL-6, IFN-α, IFN-β, IFN-γ, IL-12, and IL-18). Hence, as an adjuvant, CpG motifs can be used to stimulate Th1-type responses, and compensate/ oppose detrimental Th2-driven allergic or autoimmune responses. Nasal, oral or vaginal administration of CpG ODN results in both innate and adaptive mucosal immune responses [106, 107, 108, 109, 110]. More recently, CpG ODN along with CT was used in transcutaneous immunization protocols and elicited antigen-specific IgG and IgA Abs in the cervicovaginal tract of immunized mice [111].

3.2.3.5 Cytokines and chemokines

Cytokines or chemokines known as adjuvants can be downstream mediators of other described adjuvants (like CT, CpG), but not only. Often, the mechanisms by which they act are known (specific interactions with identified receptors), which represents a considerable asset. In addition, some cytokines and chemokines are involved in the polarization of Th cell responses; ideally, one can promote Th1-type responses for protection against intracellular pathogens or Th2-type responses required for protection against soluble Ags, allergens and toxins with the

correct cocktail of cytokines and chemokines. They can be used individually or in combination to maximize or broaden the spectrum of desired immune responses. Cytokines and chemokines are usually given as proteins. However, to compensate for their short half-life time, they have to be administered at high concentrations, which can lead to toxicity. This major limitation is nevertheless avoided when cytokines or chemokines are delivered mucosally [23, 112].

Interleukin 1. The observation that the well-known mucosal adjuvant CT induces induces IL-1 production by epithelial cells in macrophages led to investigating whether this cytokine alone could exhibit adjuvant activity for mucosal vaccines. IL-1α and β have thus been tested intranasally with a soluble protein Ag and were shown to trigger strong serum IgG, vaginal IgG and IgA Ab responses. IL-1 as adjuvant mimicked the Th2-related profile of IgG subclass Ab responses induced by CT as well as a DTH response [81] (Table 3).

Interleukin 6 and IL-12. IL-6 is known to play a role in the differentiation of IgA-producing B cells. It is also a major cytokine produced by APC in response to a number of stimuli, including CT. Nasal co-administration of IL-6 with tetanus toxoid as antigen enhanced the level of antigen-specific Ab responses in the serum. The pattern of IgG subclasses induced by IL-6 as adjuvant was a Th2-like with dominant IgG1 and low IgG2a Abs. Interestingly, IL-6 failed to induce SIgA Abs. Nasal co-application of IL-12 was shown to increase serum antibody responses to a number of vaccine antigens and to promote cell-mediated immunity [22]. IL-12 could also induce SIgA Abs, although exact mechanisms underlying the induction of SIgA by this cytokine and not IL-6 remain elusive. Finally, the efficacy of IL-12 as mucosal adjuvant can be improved by co-administration with other regulatory molecules. For example, IL-12 and GM-CSF (granulocyte-macrophage colony-stimulating factor) can synergize when administered with an HIV peptide to induce strong CTL responses [92, 113]. In another study by Staats et al. [114], IL-1 in combination with Th1-inducing cytokines such as IL-12, IL-18 and GM-CSF, induced strong mucosal and systemic responses and gave rise to a mixed Th1 (CTL and IFN-γ) profile and mucosal IgA Abs (Table 3).

Fms-like tyrosine kinase receptor-3 ligand. A single intratracheal dose of the growth factor Fms-like tyrosine kinase receptor-3 ligand (Flt3 L) followed by the application of TT resulted in increased levels of specific IgA and IgG Abs in the lung of rats, most probably by increasing the number of DCs and T cells in lung tissues (Pabst, 2003) [115]. In the same manner, GM-CSF was applied on the skin to enhance the number and activation status of epidermal dendritic cells. It was shown to increase the immune response elicited by transcutaneous immunization (IgG

responses in serum, uterine lavage and bronchoalveolar lavage and IgA responses in vaginal lavage) and to enhance the protection against genital and respiratory tract chlamydial infections [116]. Along the same line, nasal co-administration of the protein Ag OVA and a plasmid DNA expressing Flt3 L (pFlt3 L) in C57BL/6 mice promoted Ag-specific secretory-IgA Abs in nasal washes, saliva, fecal secretions and systemic Ab responses, with predominantly IgG1 and IgG2b Abs, suggesting the development of an OVA-specific Th2-type response [117]. Interestingly, unlike CpG ODNs which stimulate Th1-type responses, pFlt3 L promoted Th2-type responses [117] (Table 3).

Chemokines. Chemokines are a family of structurally related glycoproteins with chemotactic activity. Lymphoctactin (Ltn) is chemotactic for lymphocytes but not for monocytes or neutrophils. Ltn is produced by natural killer (NK) cells and attracts both NK cells and $CD8^+$ T cells [118]. Nasal co-administration of OVA with Ltn in mice enhanced OVA-specific Ab responses in the serum and the mucosal secretions and Ag-specific $CD4^+$ Th1- and Th2-type cells [119]. RANTES (regulated upon activation, normal T-cell expressed, and presumably secreted) is another CC chemokine, which displays mucosal adjuvant activity for nasally co-administered protein antigen. It is characterized however by Th1 responses and predominantly plasma IgG2a and IgG3 subclass Abs [120]. Macrophage inflammatory protein-1alpha (MIP-1α) and MIP-1β are highly homologous CC chemokines produced by a variety of host cells in response to various external stimuli and share affinity for CCR5. MIP-1α also binds CCR1 as well as CCR3 in the mouse while MIP-1β is a ligand for CCR8. Nasal delivery MIP-1α promoted strong plasma IgG Abs as well as mucosal and systemic CMI responses to co-administered Ag, while MIP-1β was a less effective adjuvant for plasma IgG Abs and failed to induce CMI responses. However, MIP-1β supported higher levels of mucosal SIgA Abs [121] (Table 3).

Mucosae-associated epithelial chemokine (MEC) also known as CCL28, is expressed in a variety of human and mouse tissues, and it appears to be predominantly produced by epithelial cells. It was originally found to bind to CCR10 but also to CCR3 [122, 123]. CCL28 is selectively chemotactic for IgA Ab-secreting cells (ASC) (but not IgG or IgM) from both intestinal and non-intestinal lymphoid and effector tissues [124]. Interestingly, CCL28 is not only a chemoattractant for mucosal cells but also displays antimicrobial activities against $Gram^+$ and $Gram^-$ bacteria, in mice as well as in humans [125]. Studies in HIV-infected or -exposed breast-feeding women demonstrated that CCL28 was augmented in breast milk and saliva as well as $CCR3^+$ and

CCR10$^+$ B cells lymphocytes. This augmentation of CCL28 in breast milk was correlated with higher survival rates in brest-fed children of HIV-infected mothers. When CCL28 was administered intramuscularly along with recombinant VSV antigen i.p. in BALB/c mice, rectal IgA-secreting B cells were significantly increased, suggesting CCL28 can modulate mucosal IgA immune responses [126]. Further studies are required to comprehend the scope of immune activities of CCL28 as an adjuvant.

	Nasal Antigen +	Serum Abs		Mucosal IgA	Th1/Th2
		IgG1	IgG2a		
Chemokine	LpTn	++	++	+++	Mixed
	RANTES	+	+++	+++	Th1>Th2
	MIP-1α	++	++	----	Th1>Th2
	MIP-1β	+++	----	++	Th1<Th2
	pFlt3 L	+++	----	+++	Th2
	CCL28	?	?	+++	?
Cytokine	IL-12	+++	+++	+++	Th1>Th2
	IL-6	++	+	----	Mixed
	IL-4	++	----	----	?
	IL-1	+++	+/-	+++	?

Table 3. Summary of immune responses induced by cytokines and chemokines as mucosal adjuvants.

3.2.3.6 Saponins and derivatives

Extracts of the bark of a South American tree, *Quillaja saponaria Molina*, such as Quil A saponin, have been shown to be potent adjuvants. QS-21 is a pure fraction of Quil A saponin with low toxicity; it is a highly purified complex triterpene glycoside [127]. QS-21 promotes both humoral and CMI responses when added to systemic vaccine formulations and has now been tested in several parenteral vaccine formulations [128, 129]. QS-21 can also acts as a mucosal adjuvant when given to mice by the oral route. However, different doses of QS-21 influence the outcome of the immune response. A low dose QS-21 given orally promoted mucosal SIgA Ab responses, while a high dose did not result in the development of SIgA Abs but in a stronger Th1-type response [130]. To this day, the mechanisms by which saponin derivatives promote mucosal immunity and control CD4$^+$ Th cell subsets differentiation remain to be determined.

3.2.3.7 Alpha-galactosylceramides (α-GalCer)

Alpha-galactosylceramide is a glycolipid extracted from marine sponge. αGalCer is a ligand of Va24 chain invariant on NKT cells and is presented by CD1d MHC molecules on APCs. It was initially used for its anti-tumor potential [131]. αGalCer-pulsed monocyte-derived cells infected with *Mycobacterium tuberculosis* were able to activate CD1d-restricted NKT cells and induced the production of proinflammatory cytokines, the lysis of infected cells, and the release of antimycobacterial peptides (granulysin) [132]. IL-4 was required for NKT cells to produce IFN-γ in response to αGalCer [133]. Ko et al. were the first to show in 2005 that αGalCer is a nasal adjuvant for co-administered protein antigen [134]. As a nasal adjuvant, it resulted in systemic and mucosal Ab responses with the presence of secretory IgA Abs in nasal and lung washes, in the development of CTLs, as well as in a mixed Th1 and Th2 immune response characterized by the production of IFN-γ and IL-4 cytokines. Nasal immunization with an influenza antigen with αGalCer induced protection against influenza virus challenge in BALB/c mice and complete protection against tumor challenge in C57BL/6 mice. Moreover, unlike the enterotoxin adjuvants CT and LT, αGalCer did not redirect Ag to the CNS [135]. In humans, αGalCer analogues are used to pulse DCs and studies have suggest it can serve as adjuvant in immunotherapy against cancer and infectious diseases [136].

Summary on mucosal vaccines and mucosal adjuvants: Considering the general agreement that mucosal vaccines can provide a better protection against mucosal diseases, it is clear that a ridiculously low number of mucosal vaccines are currently licensed wordwide. Furthermore, despite tremendous research efforts and interest for mucosal adjuvants, only systemic vaccines with aluminium salts, the oil-in-water emulsion MF59 or virosomes were approved. Enterotoxins lacking enzymatic activities raised some hope for their use as nasal adjuvant, which was rapidly dismissed by their potential to target brain tissues. Alternative vaccine delivery routes, such as the sublingual or transcutaneous routes may offer safer alternatives to oral or nasal vaccines. There is also still a need for new adjuvants which, alone or in combination with cytokines or other adjuvants, could improve vaccine efficacy but not their toxicity. TLR agonists like CpG or the NK ligand αGalCer are among some of the most discussed molecules. Nonetheless, the field of mucosal vaccines remains in need of new adjuvants and delivery systems.

3.3 Anthrax

3.3.1 *Bacillus anthracis* infection and anthrax disease

Anthrax is a disease caused by *Bacillus anthracis*, a large Gram-positive, spore-forming, non-motile bacillus (Figure 7). The disease is traditionally a zoonotic disease and humans generally become infected by contact with infected animals or contaminated animal products via the cutaneous route and only very rarely, via the respiratory or gastrointestinal routes. Cutaneous anthrax is mild and can be treated with antibiotics. Gastrointestinal or inhalational anthrax, if left untreated, usually leads to fatal systemic disease with a mortality rate approaching 100 percent [137].

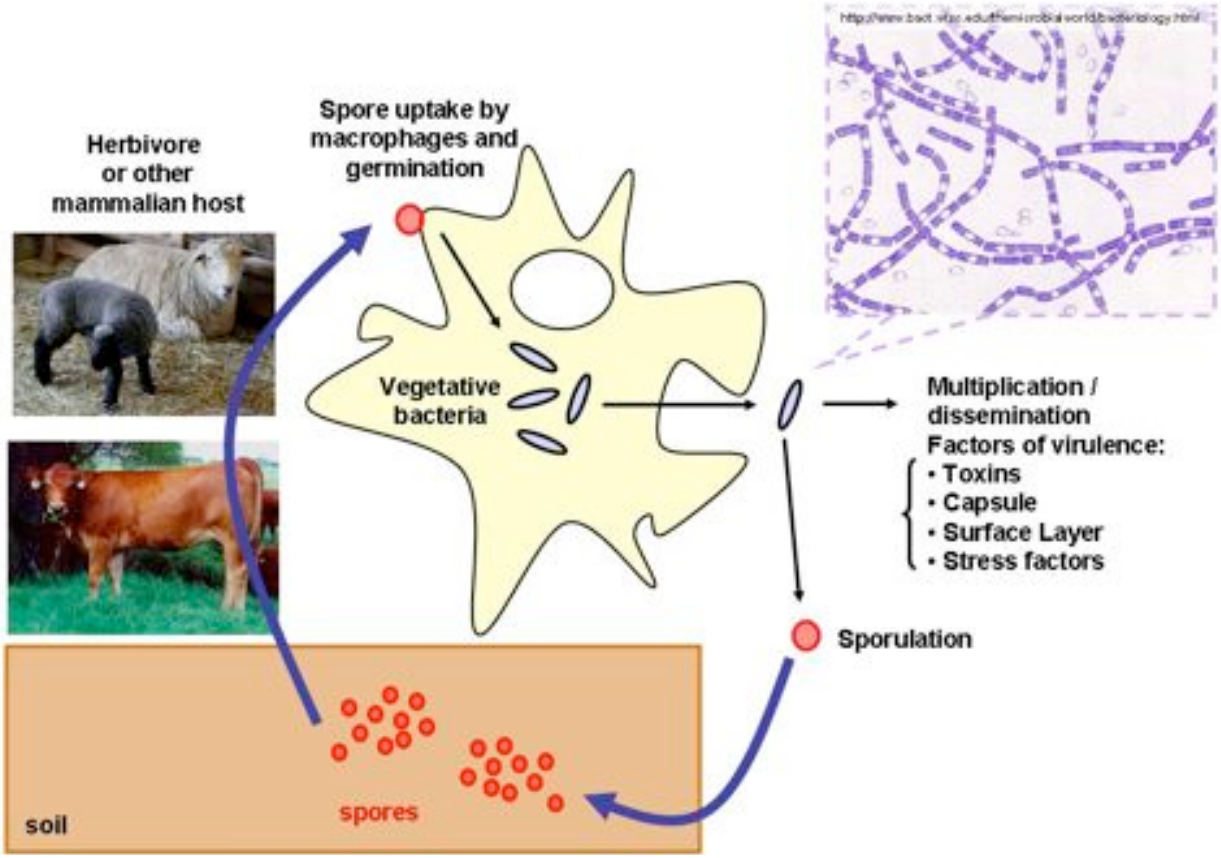

Figure 7. *Bacillus anthracis* life cycle.

Bacillus anthracis is a large gram^{+}, non-motile, aerobic, and facultative anaerobic, spore-forming bacteria. Anthrax is primarily a disease of domesticated and wild animals, particularly herbivorous animals. Humans become infected incidentally when brought into contact with infected animals. This can occur through the ingestion of undercooked meat (gastrointestinal anthrax), through cuts in the skin (cutaneous anthrax) or even through inhalation of spores present in the fur or hides of animals (woolsorters' disease leading to inhalational anthrax). The bacterium persists in the soils in the form of endospores for years. After being consumed by an animal they germinate, changing from the resistant form into the growing and dividing vegetative form. Protected by their capsules of poly-D-glutamic acid, bacilli multiply and start to release virulent toxins. While the capsule is the outermost element of the cell wall, *B. anthracis* bacilli may also possess an S-layer directly around the cell wall. It appears that the capsule and S-layer have cumulative effects against host immune defense mechanisms. Finally, *B. anthracis* bacilli are equipped with transcription factors, which help them survive under stressful conditions during their vulnerable vegetative state. [Adapted from Mock et al. [138]].

Anthrax has recently gained much attention because of the potential use of *Bacillus anthracis* spores as biological warfare agents. Inhalational anthrax is the most lethal form. The human LD50 (dose sufficient to kill 50% of infected persons) is estimated to be 2,500 to 55,000 inhaled spores [139]. *B. anthracis* possesses two major determinants of virulence: an antiphagocytic poly-g-D-glutamic acid (PGA) capsule [140], and two protein exotoxins, the lethal and the edema toxins [141]. The capsule is believed to play a role in establishment phases of the infection by protecting vegetative bacterial cells against phagocytosis and antibacterial proteins of the host sera. Both anthrax toxins are considered the most important anthrax factor contributing disease symptoms, by impairing the host defenses.

3.3.2 Mode of action of anthrax toxin complexes

Two anthrax toxin receptors have been recently identified: the anthrax toxin receptor/tumor endothelial marker 8 (ATR/TEM8) [142] and the related capillary morphogenesis gene 2 (CMG2) protein [143]. TEM8 is reported to be overexpressed during tumor angiogenesis, whereas CMG2 is more widely expressed in normal tissues. Anthrax toxins are composed of three proteins acting in binary combination. The binding subunit is termed protective antigen (PA), for its ability to elicit a protective immune response against anthrax; the enzymatic subunits, which interact with PA, are named lethal factor (LF); and edema factor (EF). Separately, none of these proteins is toxic. However, when combined and injected intravenously, PA and LF (LeTx) provoke death in animals [144], while PA + EF (EdTx) induces an edema in the skin after intradermal injection [145]. These two observations led to their respective names, lethal toxin and edema toxin. Nevertheless, EdTx is also a virulent factor shown to cause severe pathologies, like adrenal necrosis and death in BALB/cJ mice [146, 147].

Once PA binds to ATRs, it is cleaved by a furin, which leads to the release of PA20. The remaining PA63 receptor-bound fragment heptamerizes and is then able to bind LF and EF. The hetero-oligomeric complex associates with specialized cholesterol and glycosphingolipid-rich microdomains of the plasma membrane or lipid rafts and is then internalized. Some studies have demonstrated that the low-density lipoprotein receptor-related protein 6 (LRP6), co-receptor of TEM8 and CMG2, promotes receptor-mediated endocytosis of anthrax toxins into cells [148]. Recently, others have shown LRP6 was not necessary for PA internalization [149]. After acidification of the endosome, PA63 heptamer forms a pore through which LF and EF translocate. LF and EF then reach their cytosolic targets [138, 150]. The N-terminal 250 amino-acid regions of EF and LF have a similar sequence homology and are involved in PA binding and complex internalization. The other parts of EF and LF are involved in the catalytic activities. LF mature protein contains 776 residues and is a zinc-metalloprotease that cleaves the amino N-terminus of mitogen-activated protein kinases (MAPKKs), Mek1 and Mek2 [138]. The MAPK pathway relays environmental signals to the transcriptional machinery in the nucleus and thus modulates gene expression via a burst of protein phosphorylation. LeTx activities involve the inhibition of the production of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 and macrophage cell death, which could account for shock. The 767 residues mature protein EF is a potent calmodulin (CaM)-dependent, and Ca^{2+}-dependent adenylate cyclase, with a 1000-fold higher catalytic rate than that of mammalian CaM-activated adenylate cyclases [151, 152]. It catalyzes the conversion of ATP to cyclic AMP. EdTx inhibits phagocytosis in neutrophils, T cell proliferation and cytokine production [153, 154, 155] (Figure 8).

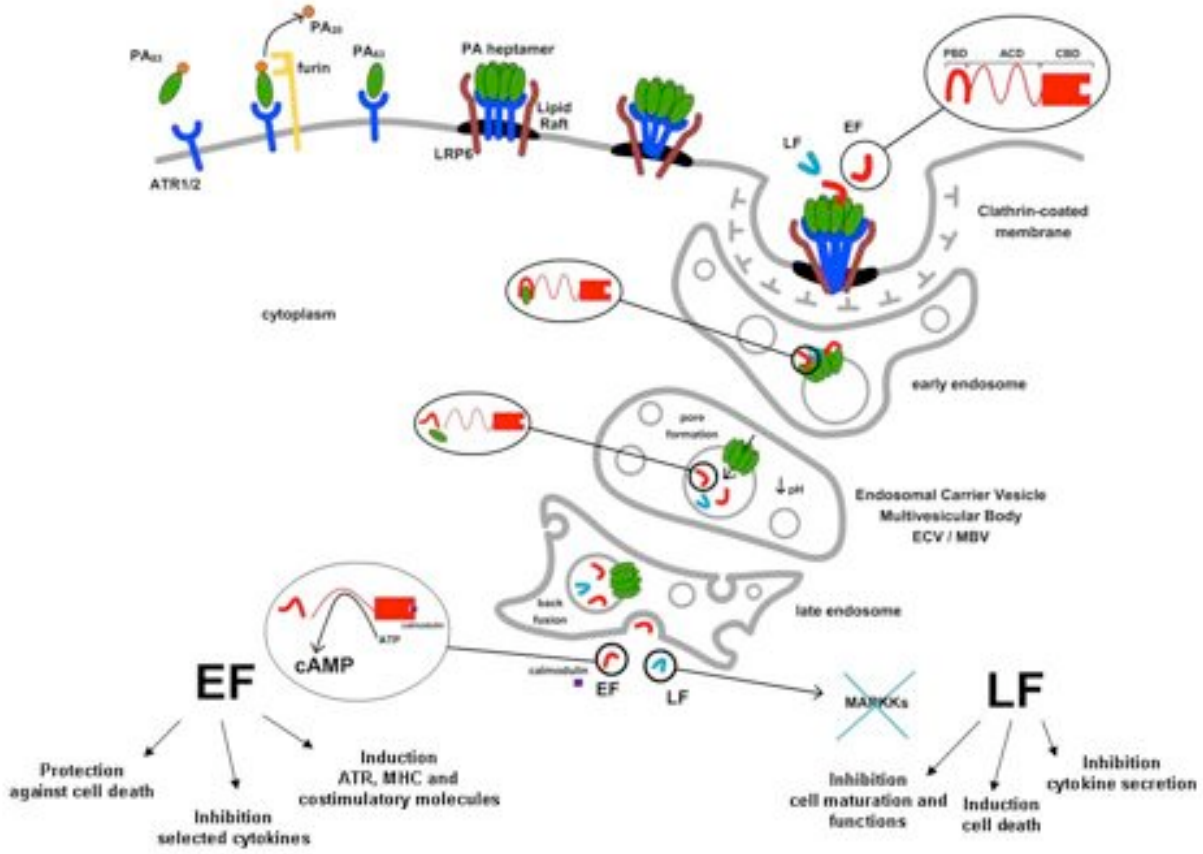

Figure 8. Anthrax toxins cell entry.

PA binds to the cell surface anthrax toxin receptors ATR (TEM8 or CMG2) and is cleaved by a furin; this leads to the release of PA20. The remaining 63 receptor-bound fragment displays the binding site for EF and LF. PA63 oligomerizes to form ring-shaped heptamers, which binds LF and EF. Upon binding of LF and/or EF, the PA63-toxins complex associates to plasma membrane lipid rafts and is then internalized by a receptor-mediated endocytosis through the clathrin-dependent pathway. The complex is transported to endosomes where the low pH triggers pore formation of the PA63 heptamer, allowing the translocation of LF/EF. As a final step, EF and LF reach the cytosol. LF is a zinc-dependent metalloprotease, which cleaves MAPKKs, Mek2 and 3. EF is an adenylate cyclase which converts ATP into cyclic AMP. [Adapted from Tournier J.N. *Contribution of toxins to the pathogenesis of inhalational anthrax*, 2007. Cell Microbiol. 9:555].

Several studies have unveiled a major role for anti-PA antibodies in the protection against anthrax [156, 157, 158]. Thus, passive transfer of polyclonal or monoclonal PA-specific IgG antibodies to mice, rabbits, guinea pigs and monkeys conferred protection to both cutaneous and inhalational forms of anthrax [156, 159]. *In vitro*, anti-PA antibodies can also protect mouse macrophages from toxicity induced by the LeTx and this provides the basis for the *in vitro* macrophage toxicity assay used as a correlate of protection [160, 161]. In addition, other studies suggest that protection against anthrax involves not only antibody to PA but also to other anthrax toxin components, like LF. Thus, both anti-PA and anti-LF antibodies were detected in the sera of individuals with histories of clinical anthrax or in individuals that had received the British

vaccine (alum precipitated, cell free filtrate of an aerobic supernatant from the non-capsulated Sterne strain of *B. anthracis*, which originally contained mostly PA but also substantial amounts of LF and EF) [162].

3.3.3 Use of anthrax toxins in immune therapy

The combination of PA plus the N-terminal 1-254 fragment of LF (LFn^{1-254}) has been used as a "molecular syringe" to introduce foreign antigens coupled to LFn^{1-254} into target cells (Figure 9). This strategy successfully delivered short peptides of bacteria, or virus [163, 164], as well as whole proteins [14, 165] in cells of BALB/c or C57BL/6 mice and the development of partially protective CTL responses. Moreover, the injection of dendritic cells treated with PA plus LFn fused to hepatitis C virus epitopes efficiently primed CTLs *in vivo* [166].

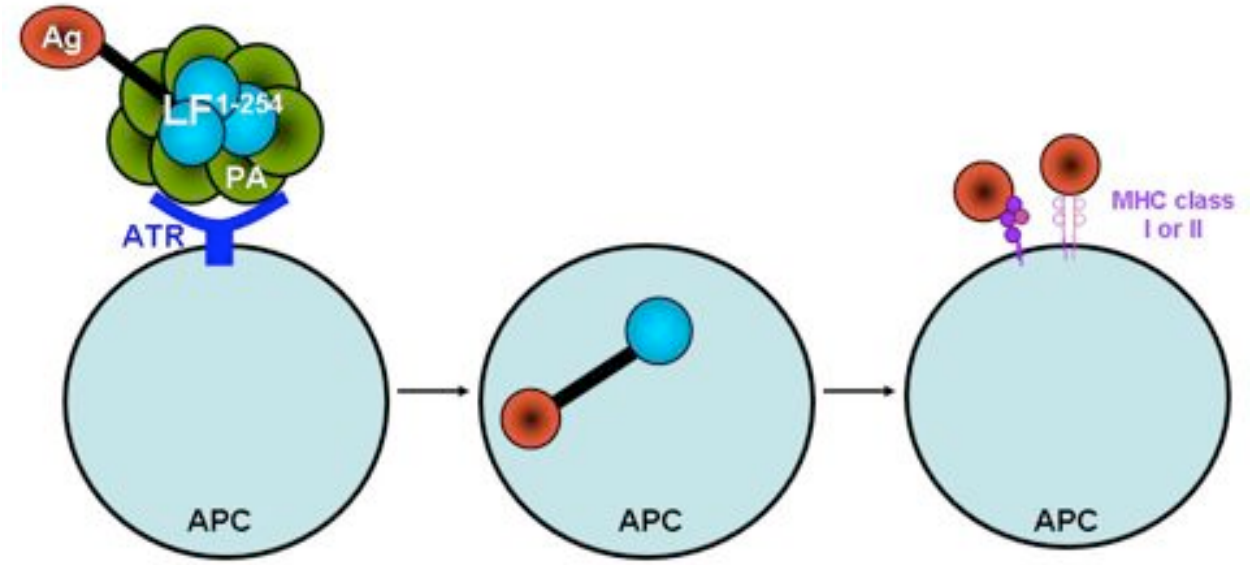

Figure 9. Anthrax toxin derivatives as antigen delivery system.
N-terminal 1-254 fragment of LF (LFn^{1-254}) binds to PA at cell surface. When LFn^{1-254} is fused to foreign antigens as various as short peptides of bacteria, or virus, or whole proteins, it can deliver such Ag into target cells, in the presence of PA, where they can be presented by a particular MHC I or MHC II molecule. This concept has been termed the "molecular syringe".

Studies demonstrated that delivery to the cytosol required an active PA [167], and the presence of a furin for cleavage of PA [168] and that presentation, which involved the classical processing for class I MHC molecules, required a functional proteasome complex [167, 169] and transporter associated with antigen processing TAP [170]. While the effective delivery of LFn fused to short epitopes (up to 33 aa) occurred in the presence of PA, Kushner et al. showed that LFn fused to green fluorescent protein (GFP) could enter the cell without PA, and that LFn fused to a variety of viral antigens (up to 550 aa long) could induce CTL without PA, but in the presence of an

adjuvant [169]. These authors hypothesize that without PA, LF could not exert its toxic activities. The PA-independent mechanism of entry of LFn remains unknown.

Summary on anthrax toxins. Anthrax toxins are responsible for most of the pathology associated with anthrax infection. PA, the binding subunit of these toxins, is the contral component of current anthrax vaccines and anti-PA Abs can protect the host against these toxins. The ability of PA to deliver the enzymatic subunits of anthrax toxin and their derivatives into cells has been exploited for the delivery of antigens and could help develop new immune therapies.

Aim of the study

4 Aim of the study

4.1 General aim

Vaccines delivered by mouth, nasal spray or drops, rectally or transcutaneously present major advantages over those given by injection (i.e., systemic route). From a practical standpoint, administration of these needle-free vaccines is painless and does not require large contingent of trained medical personnel. From an immunological standpoint, only vaccines given through mucosal and cutaneous sites will more likely promote antibody and T cell responses at the mucosal sites of entry of most pathogens. However, the development of effective and safe adjuvants has been the chief drawback in licensing new vaccines to be administered via mucosal routes. Studies with the most described experimental mucosal adjuvants, CT and LT, have helped understand that the intrinsic activity of adjuvants (i.e., their enzymatic activity) and the nature of receptor they target on host cells contribute to both their effectiveness as mucosal adjuvant and their potential toxicity. Furthermore, the recent evidence that transcutaneous immunization with enterotoxin adjuvants can promote mucosal immunity in the GI tract suggests that previously unknown homing pathways exist for induction of mucosal immunity via transcutaneous immunization.

Our work stems from our observation that nasal delivery of sublethal doses of *Bacillus anthracis* edema toxin does not inhibit, but rather enhances immune responses of nasal protein vaccine given with CT as adjuvant. This finding led to our overall hypothesis that *Bacillus anthracis* edema toxin can act as an adjuvant and promote both mucosal and systemic immunity to mucosally co-administered vaccine antigens, like ovalbumin and recombinant F1-V fusion protein of *Yersinia pestis*. We also wanted to test the hypothesis that *Bacillus anthracis* edema toxin, which binds to specific receptors (i.e., ATR1/TEM8 and ATR2/CMG2) on target cells, will promote a different pattern of immune responses than CT. The implications of the unique targeting of EdTx for the safety of EdTx-based vaccines were also addressed, as well as the efficacy of EdTx as adjuvant for transcutaneous vaccines.

4.2 Specific aims

The following specific aims were developed to address our hypothesis that *Bacillus anthracis* edema toxin can act as an adjuvant and promote both mucosal and systemic immunity to mucosally or cutaneously administered vaccine antigens:

1. Address whether the ATR-binding toxin EdTx is an adjuvant capable of promoting systemic and mucosal immunity to protein vaccines administered by the nasal route;

2. Assess whether EdTx targets the CNS after nasal delivery in mice and potential implications for safety of EdTx derivatives as adjuvants for nasal vaccines;

3. Examine innate signals involved in the immune enhancing activity of EdTx and in its ability to promote mucosal immunity;

4. Explore the potential of EdTx derivatives to act as adjuvants for vaccine formulations given by transcutaneous or sublingual application, and promote homing of immune effector cells for induction of mucosal immunity.

Results

5 Results

As an introduction to the result section, it is important to indicate that most of our findings are reported in the manuscripts listed below and attached in the Appendix. In this section our most important findings are presented with regard to the four Specific Aims underlying my PhD research. All *in vivo* immunization studies were performed with a minimum of 4 or 5 mice per group and were repeated a minimum of three times. The antibody and cytokine responses were reported as the mean + one standard deviation (SD). Statistical significance ($p < 0.05$) was determined by Student's t test and by the Mann-Whitney U test of unpaired samples. The results were analyzed using the Instat statistical program (San Diego, CA) for Apple computers. It is worth indicating that mean ELISA end-point titers displaying a difference of 2 logs consistently translated into statistical difference with p values < 0.05.

One could also question what lead to investigating the potential adjuvant activity of this virulent factor of *Bacillus anthracis.* In fact, we initially questioned if *Bacillus anthracis* EdTx would inhibit the immune response against a protein vaccine given nasally with CT as an adjuvant. In these studies mice were nasally immunized three times at weekly intervals with ovalbumin (OVA, 100 µg) with or without CT (1 µg). As shown in

Figure 10, co-administration of CT as adjuvant significantly enhanced the levels of OVA-specific IgG Abs. In addition, when 0.2 µg of EdTx were added to the vaccine at the times of immunization, the OVA-specific IgG1 Ab levels were further increased above levels achieved by CT as adjuvant.

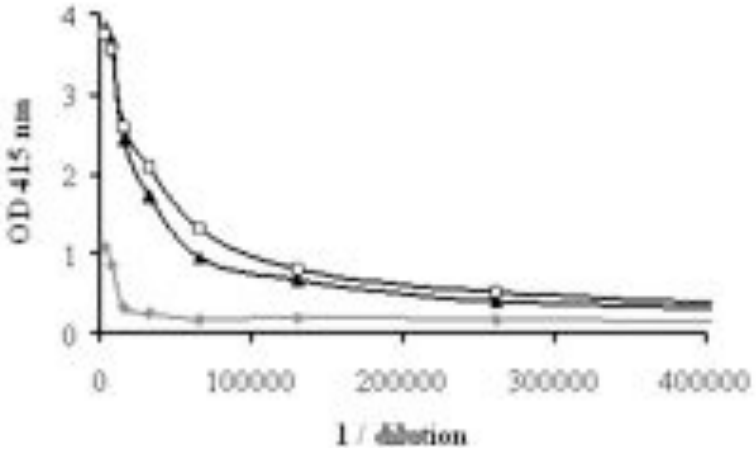

Figure 10. Serum OVA-specific IgG1 Abs after nasal CT.

Mice were immunized with 100 µg of OVA only (◆) or OVA plus 1 µg of CT (▲) or OVA plus CT plus 0.2 µg of EdTx (PA plus EF) (□). OVA-specific IgG1 Ab responses were determined one week after the last immunization in serum dilutions. The results are expressed as optical density at 405 nm.

This seminal observation and the fact that SIgA were also increased led us to hypothesize that EdTx or its derivatives could be mucosal adjuvants.

5.1 Anthrax edema toxin is an adjuvant for nasal vaccine antigens and promotes SIgA antibodies

Nasal immunization has been extensively used in animal models to evaluate the ability of candidate molecules to act as adjuvants and promote mucosal immunity. Thus, we first examined if EdTx derivative with reduced enzymatic activity was capable of enhancing plasma Ab responses to a nasally co-administered protein-Ag. As depicted in Figure 11, groups of mice nasally administered with OVA plus 1 µg of EdTx (1 µg of PA plus 1 µg of the EF S418N mutant of EF) showed increased OVA-specific IgG Ab levels than control group that received OVA alone. The adjuvant activity of the EdTx derivative was dose-dependent as 5 µg induced higher OVA-specific IgG Ab responses. More importantly, nasal administration of 5 µg of EdTx induced plasma OVA-specific IgA Abs and this dose was selected for the subsequent experiments characterizing the adjuvant activity of EdTx (Figure 11 and [171]).

As indicated earlier in the Introduction section (and Figure 4), the ratio of IgG1 to IgG2a Abs in mice provides an indirect indication of the nature of T helper subsets involved in responses. Antigen-specific IgG1 and IgG2b Abs were the dominant IgG subclasses induced by nasal immunization with the EdTx derivative and the IgG1/IgG2a ratio corresponded to a predominant Th2-type response (Figure 11). Additional support for the induction of Th2 responses was given by the fact that one week after the second immunization with OVA and EdTx, IgE Abs were significantly elevated in the plasma (Figure 11 and [171]).

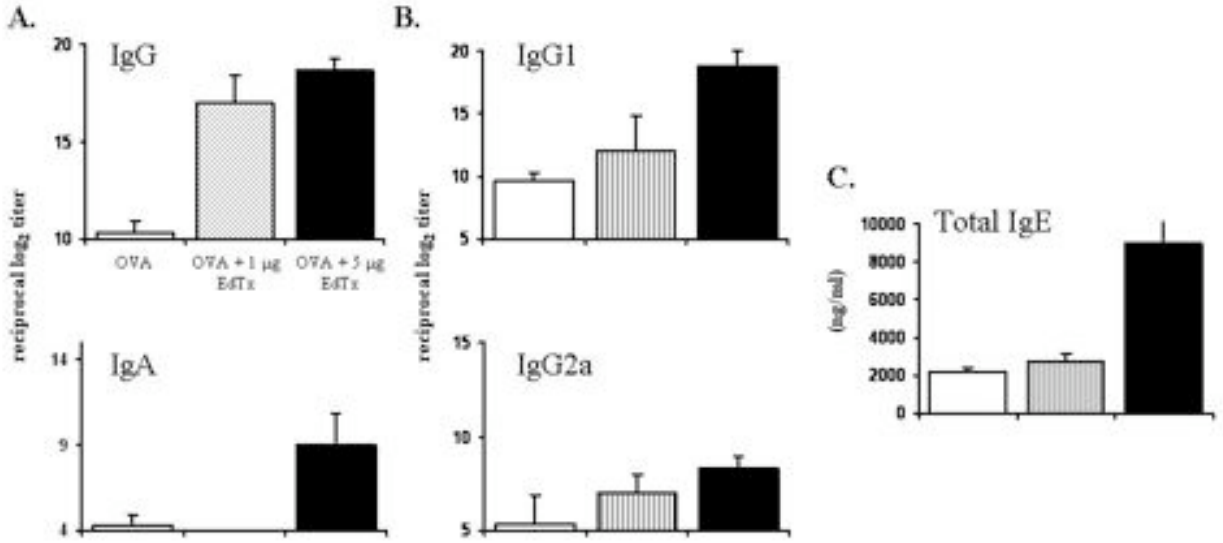

Figure 11. Serum OVA-specific Abs after nasal immunization with EdTx.

A. Serum OVA-specific isotype Ab reponses after nasal EdTx. The effect of the dose of EdTx on OVA-specific Ab response is represented by OVA-specific IgG Abs. Mice were immunized three times at weekly intervals with 100 µg of OVA only (□) or OVA plus 1 µg (⊡) or 5 µg (■) of EdTx (5 µg PA plus 5 µg EF S414N). OVA-specific IgA Ab responses were determined one week after the last immunization. B. Plasma levels of OVA-specific IgG subclass Abs were determined one week after the last immunization. C. Plasma samples were collected one week after the second immunization (day 14) and examined for their content of total IgE Ab levels. Mice were immunized three times at weekly intervals with 100 µg of OVA only (□) or OVA plus 5 µg of PA (▥) or OVA plus 5 µg of EdTx (5 µg PA plus 5 µg EF S414N) (■). Plasma levels of OVA-specific Abs were determined one week after the last immunization. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from three experiments and four mice / group.

After demonstrating that nasal EdTx augments systemic Ab immune response, we asked whether EdTx could act as a mucosal adjuvant and thus, trigger Ab responses in mucosal compartments. Nasal administration of EdTx along with OVA induced OVA-specific IgG and IgA Abs in the saliva and OVA-specific IgA in the vaginal lavages. OVA alone or OVA plus PA failed to induce OVA-specific Abs in the same compartments. In addition, nasal EdTx induced significant levels of OVA-specific IgA Abs in the fecal extracts, although these responses were lower than those induced by CT (Figure 12 and [171]).

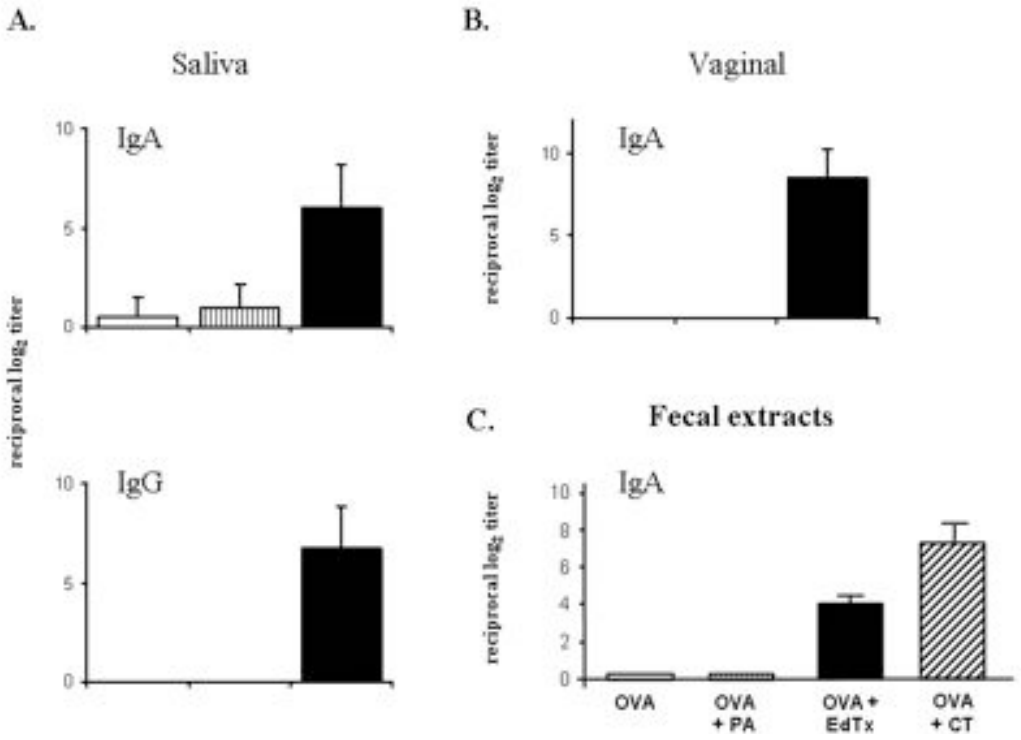

Figure 12. Mucosal SIgA Ab responses following nasal immunization with EdTx.
Mice were immunized three times at weekly intervals with 100 µg of OVA only (□) or OVA plus 5 µg of PA (▥) or OVA plus 5 µg of EdTx (5 µg PA plus 5 µg EF S414N) (■). Saliva (A) and vaginal washes (B) were collected two weeks after the last immunization. The Ab levels are expressed as the reciprocal $\log_2$ titer ± one SD from five separate experiments and four mice / group / experiment. Fecal extract (C) were collected two weeks after the last immunization with OVA only (□), or OVA plus PA (▥), or OVA plus 5 µg of EdTx (■) or OVA plus 1 µg CT (◪). The SIgA Abs levels were expressed as the reciprocal $\log_2$ titer ± one SD from three separate experiments and four mice / group / experiment.

5.2 Anthrax edema toxin is an adjuvant for its binding subunit and promotes anti-PA antibodies, which protect macrophages against LeTx-induced death

The development of Abs to PA was shown to be crucial for protection against anthrax infection. We wondered if, like the potent mucosal adjuvants CT and LT-I which are highly immunogenic toward their respective ganglioside-binding B subunits, EdTx could enhance immunity to its binding subunit PA itself. Therefore, we analyzed anti-PA Ab responses by ELISA in the same groups of nasally immunized mice. Nasal immunization with PA alone gave rise to low levels of PA-specific IgG and IgA Abs. The presence of EF greatly enhanced PA-specific IgG and predominantly IgG1 and IgG2b Ab levels (Figure 13) and induced IgA Ab responses. Only in mice nasally administered with EdTx twice at a week interval, PA-specific IgE Abs were detected, while the administration of PA alone did not trigger PA-specific IgE Abs (Figure 13). As for OVA-specific responses above, we examined the induction of anti-PA antibodies in

mucosal secretions. Nasal administration of EdTx induced PA-specific IgG and IgA Abs in the saliva and PA-specific IgA in the vaginal lavages (Figure 13 and [171]).

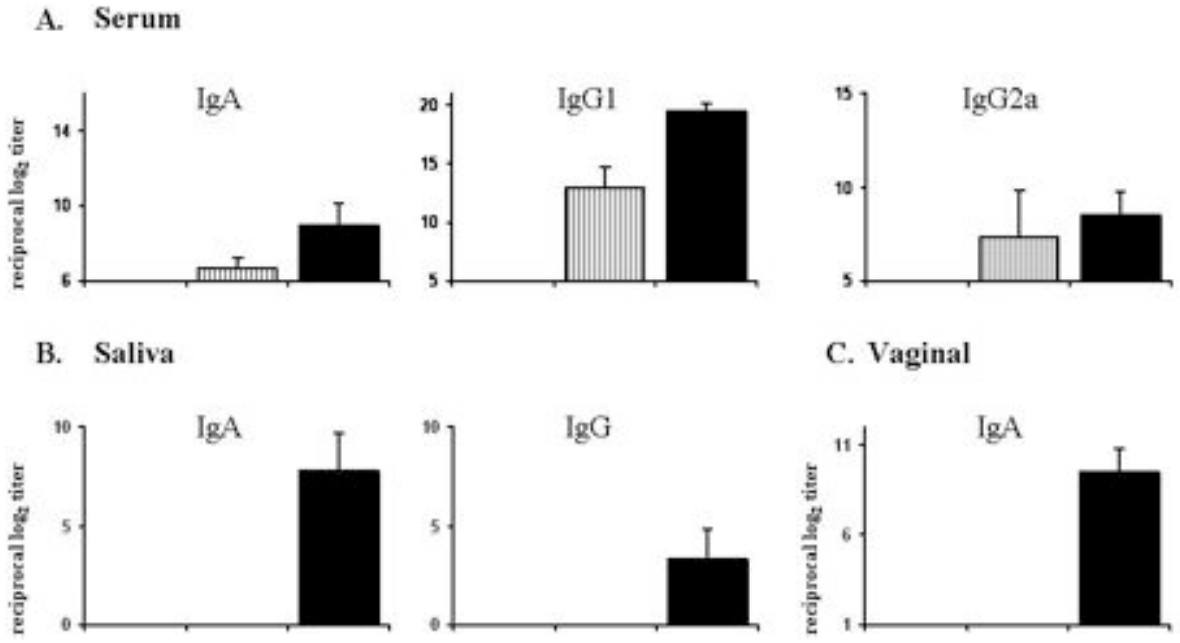

Figure 13. PA-specific Abs after nasal immunization with EdTx.
Mice were immunized three times at weekly intervals with 100 µg of OVA only (□) or OVA plus 5 µg of PA (▥) or OVA plus 5 µg of EdTx (5 µg PA plus 5 µg EF S414N) (■). PA-specific Ab isotype and IgG subclass responses were determined one week after the last immunization (A). Saliva (B) and vaginal washes (C) were collected two weeks after the last immunization. The results are expressed as the reciprocal $\log_2$ titer ± one SD from five separate experiments and four mice / group / experiment.

It was important to determine the biological significance of PA-specific Ab responses induced by nasal application of EdTx. *In vivo* protection of mice against systemic challenge with LeTx could establish the protective activity of serum Abs, but no reliable methods are available to examine the protective activity of Abs in mucosal secretions. Using a model of MØ-induced death as an *in vitro* correlate of protection, we found that plasma Abs neutralized LeTx and prevent the LeTx-induced death of MØ (Table 4). Of interest with regard to mucosal immunity, significant levels if anti-PA neutralizing Abs were found in the saliva of immunized mice (Table 4 and [171]).

	Neutralizing Abs Titers (1/Dilution)		
Immunization	Plasma	Saliva	Vaginal Washes
OVA only	ND	ND	ND
OVA plus 5 µg PA	8,192 ± 256	ND	ND
OVA plus EdTx (5 µg PA + 5 µg EF)	131,072 ± 1,024	10 ± 3	3 ± 2

Table 4. Neutralizing anti-PA Ab responses after nasal EdTx.
Mice were immunized three times at weekly intervals. Plasma samples were collected a days 21. Saliva and vaginal washes were collected one week later (day 28). Serial dilutions of each sample were added to J774 MØ cultures in the presence of LeTx. The neutralizing titers were determined as the last dilution yielding an MTT optical density equal to twice the

background value. Results are shown as neutralizing Ab titers and are expressed as the reciprocal dilutions titers ± one SE of three separate experiments, and five mice per group per experiment. ND: Below detection levels.

Edema toxin also induces Abs against edema factor: It is worth indicating that the adjuvant activity of EdTx is also associated with enhanced immunity to the enzymatic subunit EF. Indeed, nasal delivery of EF alone or EF with OVA induced low levels of EF-specific IgG Abs and failed to induce EF-specific IgA Abs (Figure 14). In contrast, mice given PA plus EF S414N exhibited high levels of EF-specific IgG and IgA in the serum, but also EF-specific IgA in fecal (Figure 14). Taken together, these findings suggest that, in addition to acting as an adjuvant for unrelated antigens, EdTx induces immunity against multiple anthrax toxin components and thus, could represent a new platform for development of more effective anthrax vaccines.

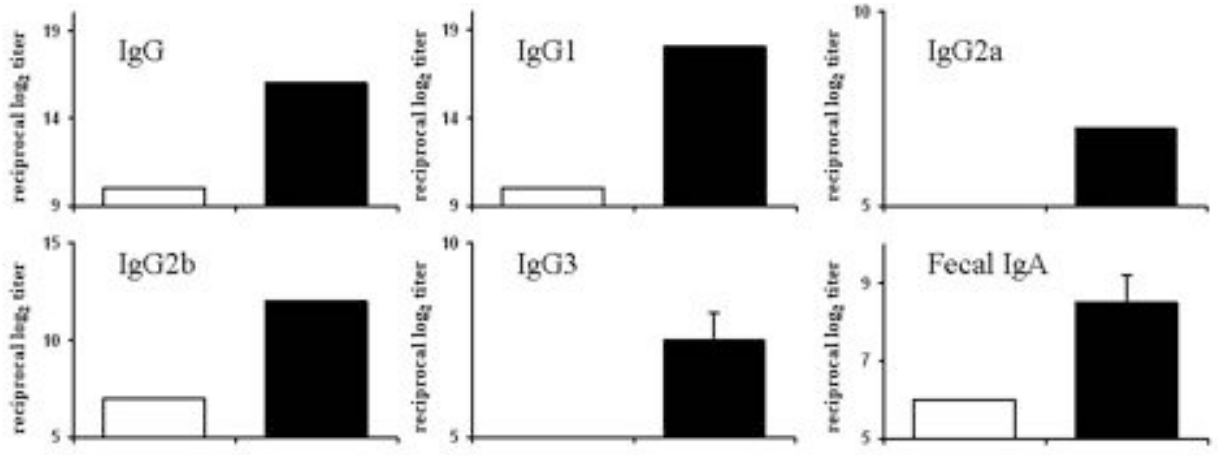

Figure 14. EF-specific Abs after nasal immunization with EdTx.

Mice were immunized three times at weekly intervals with 100 µg of OVA plus 5 µg EF (□) or OVA plus 5 µg of EdTx (5 µg PA plus 5 µg EF S414N) (■). EF-specific Ab isotype and IgG subclass responses were determined one week after the last immunization. The results are expressed as the reciprocal $\log_2$ titer ± one SD from three separate experiments and four mice / group / experiment.

5.3 EdTx as a nasal adjuvant induces antigen-specific Th1 and Th2 cell cytokine responses

While both CT and LT-I are mucosal adjuvant, the former induces strong Th2 responses while the later is associated with more balanced Th1 and Th2 responses. Previous studies have suggested that these responses were mostly controlled by the binding of their B subunits to corresponding cell surface receptors [77]. In order to determine the pattern of $CD4^+$ Th cell responses promoted by the ATR-binding EdTx, splenocytes isolated from mice immunized with OVA or OVA plus EdTx were restimulated *in vitro* for 5 days with either OVA. Cytokines

secreted by restimulated spleen $CD4^+$ T cells belonged to the Th1 pathway (IFN-γ) as well as to the Th2-arm of the T helper response with IL-5, IL-6 and IL-13 (Table 5 and [171]).

Source of $CD4^+$ T cells	Immunization	*In vitro* stimulation	Cytokines (pg/ml)			
			IFN-γ	IL-5	IL-6	IL-13
Spleen	OVA only	None	156 ± 30	ND	36 ± 1	20 ± 2
		OVA	1320 ± 49	41 ± 1	157 ± 10	36 ± 6
		PA	189 ± 10	ND	31 ± 1	20 ± 0
	OVA plus EdTx	None	267 ± 37	ND	31 ± 0	20 ± 0
		OVA	9666 ± 1524	418 ± 85	386 ± 16	188 ± 29
		PA	16550 ± 2557	1340 ± 160	192 ± 28	406 ± 78

Table 5. Antigen-specific CD4+ T cell cytokine responses after nasal EdTx.

Spleen $CD4^+$ T cells were isolated 21 days after the initial immunization with OVA only (100 µg) or OVA plus EdTx (5 µg PA + 5 µg EF S414N) and restimulated *in vitro* with OVA (1 mg/ml). After 5 days of culture, cytokine secretion was evaluated in culture supernatants by cytokine-specific ELISA and compared to those of control unstimulated cultures. The results are expressed as the mean ± one SE and are representative of four separate experiments. The same profile of responses was seen with $CD4^+$ T cells isolated from corresponding cervical lymph nodes. ND: Below detection levels.

The results presented above mostly confirm the profile of Ab responses induced by EdTx as nasal adjuvant. In order to clearly demonstrate the functional implications of the observed mixed Th1/Th2 responses, future studies could examine cytotoxic T lymphocyte responses or the delayed-type hypersensivity (DTH) responses. In this regard, we examined DTH responses in the transcutaneous immunization studies described below (for more details, see Section 5.11).

5.4 Nasal EdTx does not exhibit the safety issues associated with ganglioside-binding enterotoxins

Because there is strong evidence that enterotoxin adjuvants may have neurological effects when given via the nasal route, we examined whether EdTx could target the CNS and trigger an inflammatory response. Twenty-four hours after nasal administration of 5 µg of PA alone or along with 5 µg of EF, the olfactory nerves/epithelium (ON/E), the olfactory bulb (OB) and the brain tissues contained no trace of PA, and only minor amounts of PA were detected in the CLN, the NALT and the spleen. In contrast with PA or EdTx, the nasal administration of the same dose of CT led to a dramatic accumulation in the ON/E; even when we used a 10 times lower dose of CT (i.e. 0.5 µg), the accumulation of the enterotoxin was significant in the ON/E (Figure 15 and

[171]). Contrary to CT, nasal delivery of EdTx failed to upregulate mRNA IL-1 levels in the OB N/E and the NALT. In addition, EdTx induced much lower levels of IL-6 in nasal washes than CT (

Figure 16 and [171]). In summary, nasal EdTx does not target CNS tissues and does not induce inflammatory cytokines.

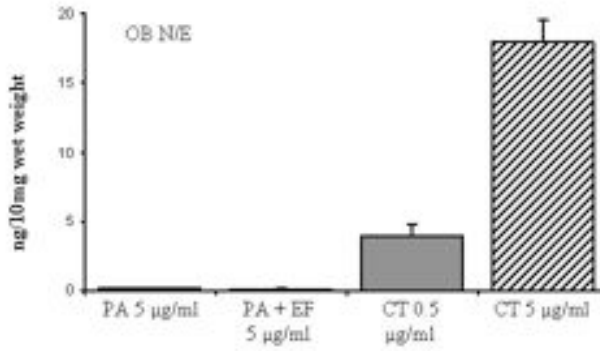

Figure 15. EdTx does not target CNS tissues.

Comparison of various acridinium labeled compounds and uptake into the olfactory nerves and epithelium 24 hrs after intranasal immunization. Groups of mice were given acridinium labeled PA (5 µg) (□), EdTx (5 µg of PA plus 5 µg of EF S414N) (■), CT (0.5 ▩ or 5 µg ◪) by the nasal route. Twenty four hours later, mice were sacrificed and the olfactory nerves and epithelium (ON/E) and olfactory bulbs (OBs) were collected, homogenized and tested for light activity as described in *Materials and Methods*. Results are expressed as mean RLU/ng of tissues ± one SD and are from two separate experiments with three mice per group.

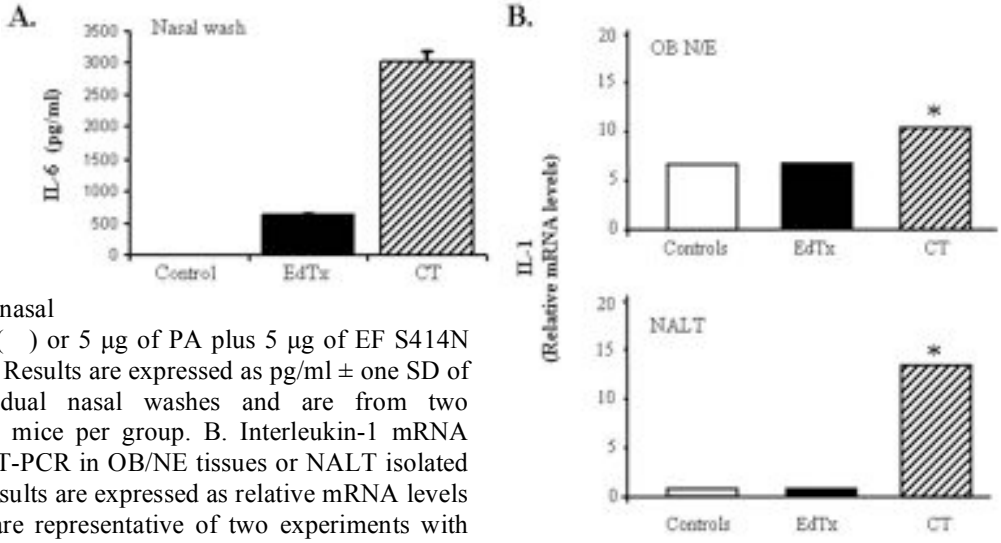

Figure 16. Inflammatory cytokine responses to nasal EdTx *in vivo*.

A. Analysis of IL-6 levels by ELISA in nasal washes of mice, 24 hours after nasal □ministration of PBS ( ) or 5 µg of PA plus 5 µg of EF S414N ■) or 1 µg of CT ( ). Results are expressed as pg/ml ± one SD of IL-6 levels in individual nasal washes and are from two experiments with three mice per group. B. Interleukin-1 mRNA analysis by real time RT-PCR in OB/NE tissues or NALT isolated from the same mice. Results are expressed as relative mRNA levels in pooled tissues and are representative of two experiments with three mice per group. *, $p < 0.05$ when compared with controls given PBS.

5.5 Role of the binding subunit PA in the nasal adjuvanticity of EdTx in vivo

Edema toxin is not a preformed toxin and is made of two distinct moieties that assemble on the surface of cells. We addressed the contribution of EdTx components in the adjuvant activity of this molecule for nasal vaccines. In our initial studies had unequivocally showed that the combination of PA and EF is necessary for the adjuvant effect of EdTx. In fact co-administration

of OVA plus PA alone or OVA plus EF alone did not enhance immune responses to OVA (data not shown).

To address the role played by the cell-targeting subunit in the adjuvant EdTx we co-administered native EF with PA-U7, devoid of furin cleavage site, or PA dFF, which is deleted Phe at amino acid 314-315 (Figure 17). The later mutant is unable to translocate LF or EF to cytosol, but is able to deliver LF or EF to endosomes. The wild-type EF was used in this series of experiments since our separate studies showed no difference between the adjuvant activity of wild-type EF and EF S414N. As vaccine antigen, we used as a fusion protein of *Yersinia pestis* F1 and V antigens (F1-V).

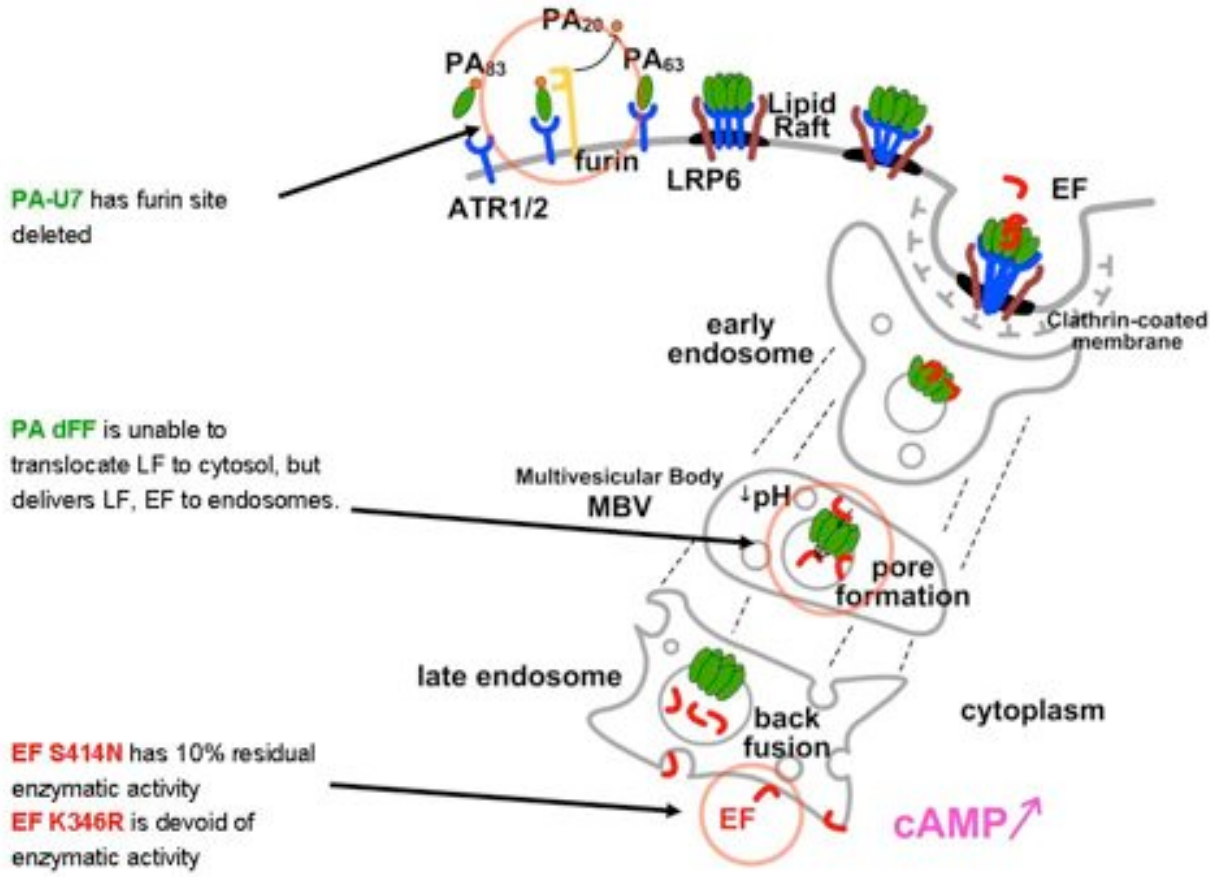

Figure 17. Native *Bacillus anthracis* toxin subunits and mutant derivatives.

PA-U7 has the furin cleavage site deleted, while PA dFF contains a site deleted for the two Phe at amino acid 314-315, which makes it unable to translocate LF or EF to cytosol, but able to deliver LF or EF to endosomes. The mutant EF protein contains a S414N mutation and displays ~20-fold less adenylate cyclase activity than the wild-type EF (EF). EF K346R is a mutant of wild-type EF devoid of enzymatic activity.

Co-administration of EF plus PA-U7 failed to significantly increase the F1-V-specific IgG or IgA Abs. In the other hand, nasal application of EF plus PA dFF, gave rise to significant levels

of F1-V-specific IgG Abs in both the plasma and mucosal secretions. However, the later combination also failed to induce IgA Abs (Figure 18).

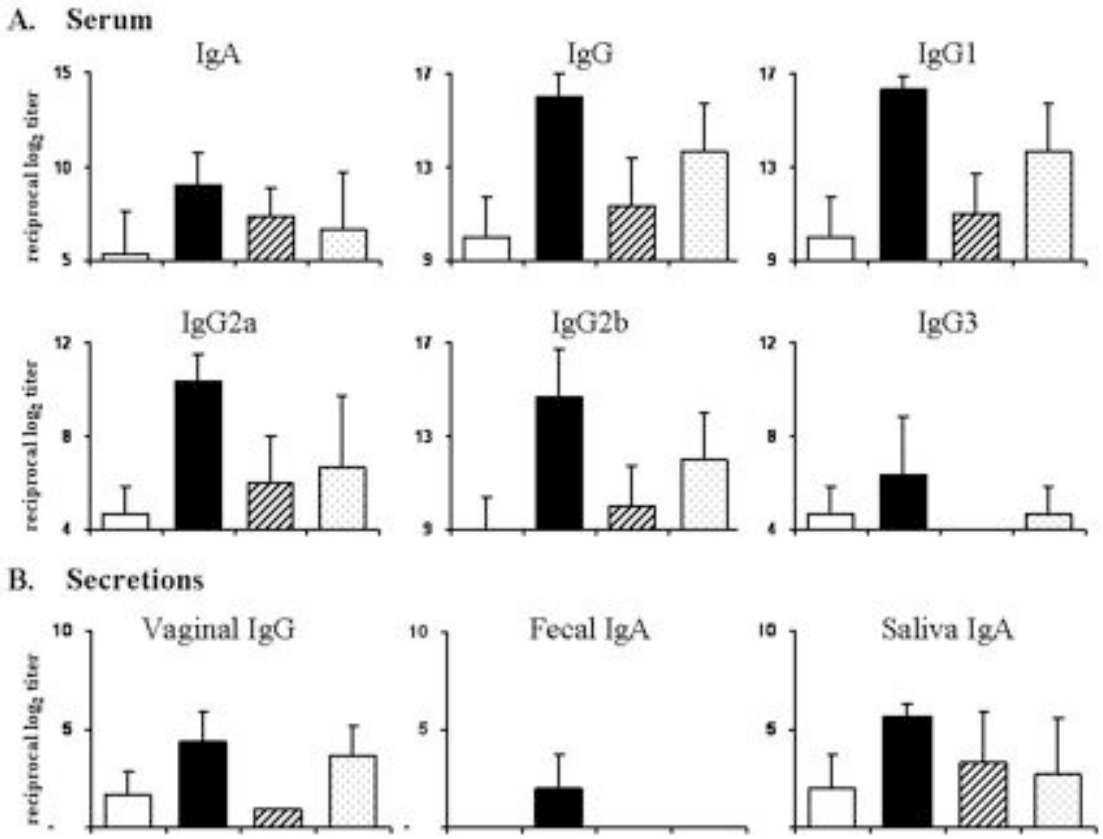

Figure 18. F1-V-specific Abs after nasal immunization with PA mutants and wild-type EF.
A. Serum F1-V-specific isotype Ab reponses after nasal EdTx derivatives. Mice were immunized three times at weekly intervals with 20 µg of F1-V only (□) or F1-V plus 2 µg PA plus 2 µg EF (■) or F1-V plus 2 µg PA-U7 plus 2 µg EF (▨) or F1-V plus 2 µg PA dFF plus 2 µg EF (⊡). F1-V-specific IgA, IgG and IgG-subclass Ab responses were determined one week after the last immunization. B. Mucosal Ab levels of F1-V-specific IgA and IgG Abs in vaginal and fecal secretions and saliva were determined two weeks after the last immunization. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from three experiments and four mice / group.

We also asked how the use of PA-U7 or PA dFF would affect the immune response to PA itself. Interestingly, anti-PA Ab responses mirrored anti-F1-V responses indicating tha same mechanism(s) control(s) the enhanced immune responses to these antigens by co-administration of EF (Figure 19).

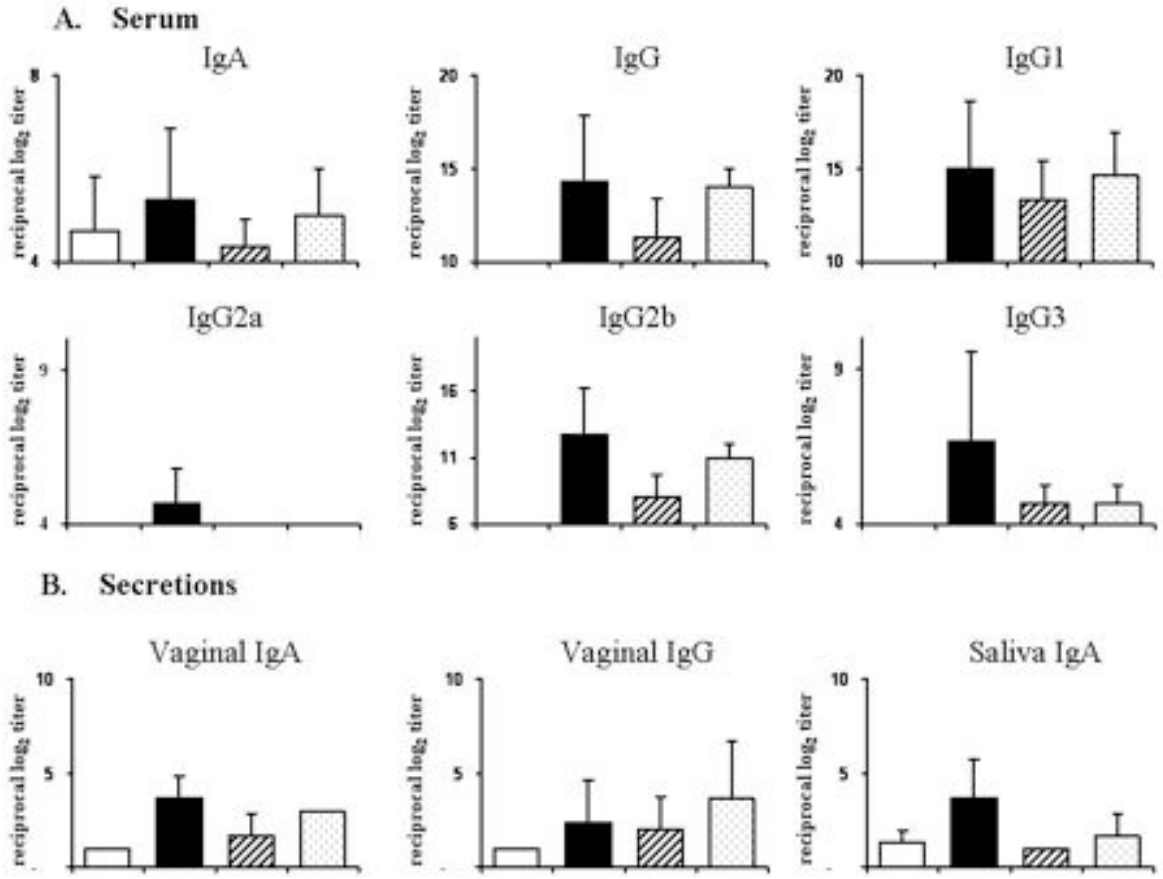

Figure 19. PA-specific Abs after nasal immunization with PA mutants and wild-type EF.

A. Serum PA-specific isotype Ab reponses after nasal EdTx derivatives. Mice were immunized three times at weekly intervals with 20 µg of F1-V only (□) or F1-V plus 2 µg PA plus 2 µg EF (■) or F1-V plus 2 µg PA-U7 plus 2 µg EF (◪) or F1-V plus 2 µg PA dFF plus 2 µg EF (⊡). F1-V-specific IgA and IgG-subclass (IgG1 and IgG2a) Ab responses were determined one week after the last immunization. B. Mucosal Ab levels of F1-V-specific IgA and IgG Abs in vaginal and fecal secretions and saliva were determined two weeks after the last immunization. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from three experiments and four mice / group.

5.6 An enzymatically active EF is needed for the nasal adjuvanticity of EdTx in vivo

The experiments summarized above have shown that both wild-type EF and the EF S414N mutants are adjuvants for nasal vaccines and that these two forms of EF lead to similar immune responses to either the co-administered vaccine antigen or PA itself. Studies with other enterotoxins have shown that CT or LT-I mutant lacking enzymatic activity can act as nasal adjuvants [172, 173]. Therefore, we asked if a mutant EF devoid of adenylate cyclase activity would enhance the immune responses to nasal vaccines. In contrast, with mice immunized three times at weekly intervals with 20 µg of F1-V plus EdTx (2 µg PA plus 2 µg EF), those co-administered

of PA plus EF K346R failed to develop F1-V-specific Ab responses (Figure 20). The lack of adjuvanticity of EF K346R was also observed for anti-PA Ab responses, which completely mirrors anti-F1-V responses. Thus, as seen for Ab responses to F1-V, co-administration of PA plus the enzymatically inactive EF K346R failed to enhance anti-PA Ab responses (data not shown).

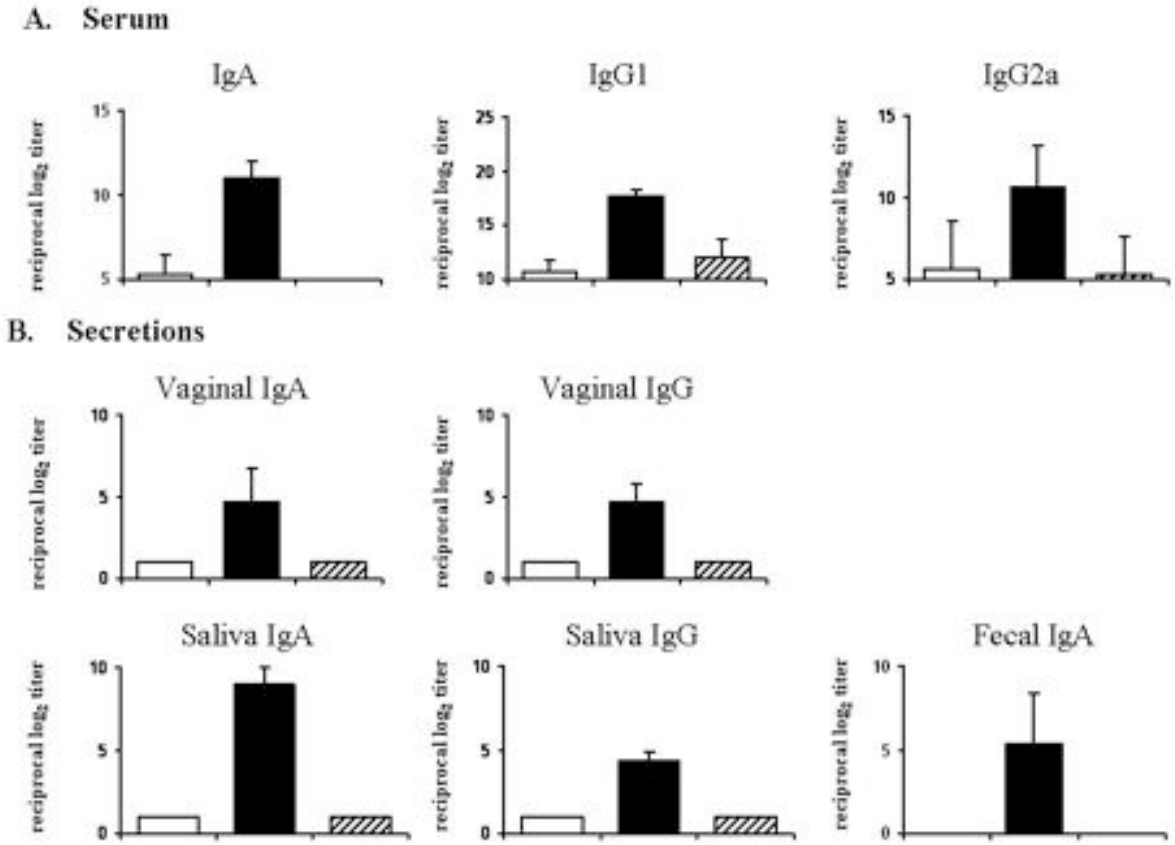

Figure 20. F1-V-specific Abs after nasal immunization with wild-type PA and EF mutants.

A. Serum F1-V-specific isotype Ab reponses after nasal EdTx derivatives. Mice were immunized three times at weekly intervals with 20 µg of F1-V only (□) or F1-V plus 2 µg PA plus 2 µg EF (■) or F1-V plus 2 µg PA plus 2 µg EF K346R (◪). F1-V-specific IgA, IgG and IgG-subclass Ab responses were determined one week after the last immunization. B. Mucosal Ab levels of F1-V-specific IgA and IgG Abs in vaginal and fecal secretions and saliva were determined two weeks after the last immunization. The results are expressed as the reciprocal log_2 titers ± one SD and are from three experiments and four mice / group.

5.7 Involvement of innate immunity components in edema toxin adjuvanticity

5.7.1 Proinflammatory cytokines do not play a major role in the adjuvant activity of EdTx derivatives

The classical mucosal adjuvant CT is known to induce proinflammatory cytokines including IL-1 and IL-6. Since nasal EdTx S414N failed to induce proinflammatory cytokines *in vivo*, we

further assessed the effects of EdTx on cytokine secretion by MØ cell lines. Consistent with previous reports, CT induced high levels of IL-6 responses in treated cells and increased costimulatory molecule expression by MØ *in vitro* (Figure 21 and [171]). The IL-6 responses to EdTx S414N stimulation were ~10-fold lower than those measured in culture supernatants of J774 MØ cultured in the presence of CT (Figure 21). The use of wild-type EdTx (PA plus EF) rather than EdTx S414N (PA plus EF S414N) did not significantly affect the level of IL-6 secretion (data not shown).

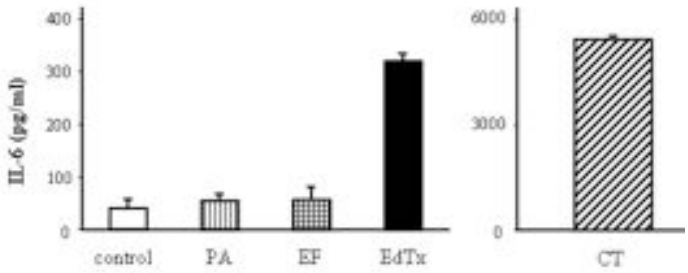

Figure 21. *In vitro* IL-6 cytokines after EdTx treatment in macrophages.

J774A.1 (or RAW264.7) mouse macrophages were treated for 24 or 48 hours in the presence of PA (5 µg/ml) (▥), EF S414N (5 µg/ml) (▦), EdTx (5 µg/ml PA + 5 µg/ml EF S414N) (■), or CT (1 µg/ml) (▨). Culture supernatants were collected and IL-6 secretion was measured by ELISA. Results are from four separate experiments. ($p < 0.05$).

Consistent with the failure of EdTx to induce proinflammatory cytokines *in vivo*, same levels of PA-specific antibody responses induced in wild-type control mice and IL-1α/β KO mice nasally immunized EdTx (Table 6). Similar results were obtained after nasal immunization with EdTx S414N (data not shown). Because of the central role played by IL-6 and IL-1 in the adjuvant activity of CT, our results suggest that EdTx derivatives may represent a new class of mucosal adjuvants for the induction of protective immunity against PA and anthrax toxins.

	Plasma				Vaginal Washes	Fecal Extracts
Treatment	IgA	IgG1	IgG2a	IgG2b	IgA	IgA
EdTx	10.1 ± 1.7	19.0 ± 0.8	11.0 ± 0.8	16.0 ± 1.1	5.8 ± 1.1	6.0. ± 1.5
EdTx + anti-NGF	10.7 ± 2.5	17.7 ± 1.1	11.7 ± 1.5	14.7 ± 0.6	6.0 ± 2.0	5.0 ± 0.6
EdTx (IL-1 α/β KO)	10.5 ± 1.5	17.0 ± 1.0	9.3 ± 2.3	14.7 ± 1.1	5.5 ± 1.7	5.3 ± 1.2

Table 6. Role of innate cytokines on anti-PA Ab response following nasal immunization with EdTx.

Normal C57BL/6 or IL-1α/β double KO mice were nasally immunized with three times at weekly intervals with 5 µg of EdTx (5 µg PA + 5 µg wild-type EF). Some groups of mice were given 50 µg of anti-NGF Ab at the time of immunization. The results are expressed as the log2 titers ± one SD and are from five mice /group. No significant difference was seen between the groups.

5.7.2 Effect of EdTx subunits and mutant derivatives on costimulatory molecule expression and antigen uptake by macrophages

The expression of costimulatory molecules and the ability to take up antigens are critical innate responses for the development of adaptive immunity. Our initial studies compared the effect of EdTx S414N (PA and EF S414N) and CT on the expression of costimulatory molecules (i.e., CD40 and CD86) by J774 MØ. Both CT and EdTx S414N significantly enhanced the expression of costimulatory molecules by J774 MØ and no significant difference was seen between the effects of the two toxins/adjuvants (Figure 22 and [171]).

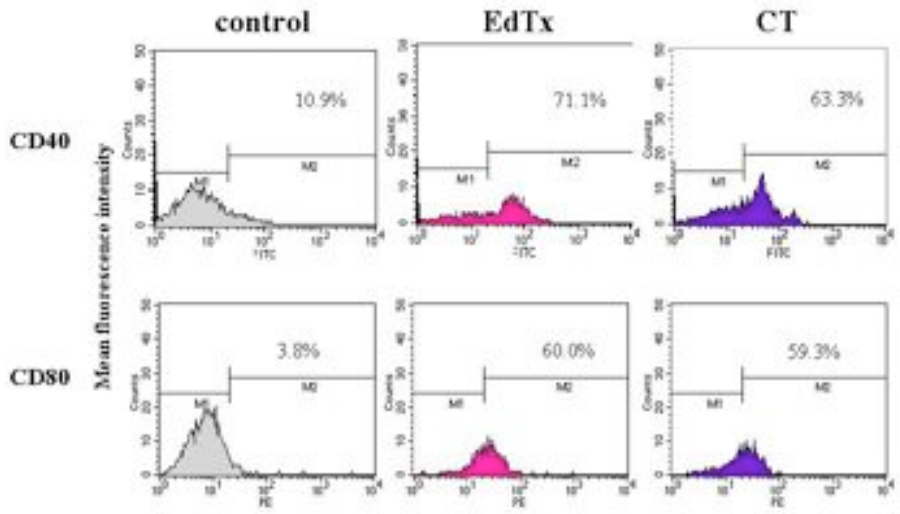

Figure 22. *In vitro* costimulatory molecule expression on macrophages after EdTx treatment.
J774A.1 mouse macrophages were plated on a 24-well plate at 10^6 cells/ml and treated for 24 hours with EdTx (2 µg/ml) or CT (1 µg/ml). The surface expression of costimulatory molecules CD80 and CD40 was assessed by flow cytometry. Results are from four separate experiments. ($p < 0.05$).

In order to understand the role of the subunits of EdTx in its adjuvanticity, we next used two mutants of the binding subunit PA, one lacking the furin site (PA-U7) and the other unable to translocate EF to the cytosol (PA dFF E308D), and a mutant of EF (EF S414N) with 10% of residual activity compared to the EF (Figure 17). Two macrophage cell lines (RAW264.7 and J774A.1) were treated for 24 hours with different combinations of mutants or wild-type PA and EF. In either cell lines, only an EdTx containing a functional PA was able to induce or upregulate the expression of CD80 and CD86. The magnitude of adenylate cyclase activity did not seem to play a role in the modulation of CD80 and CD86 expression since both the mutant of EF and the wild-type EF induced similar levels of expression of CD80 or CD86 (Figure 23). Similar results were obtained when spleen cells were used instead of the macrophage cell lines (data not shown). Future studies will determine whether an EF moiety lacking enzymatic activity can modulate co-stimulatory molecule expression on APCs.

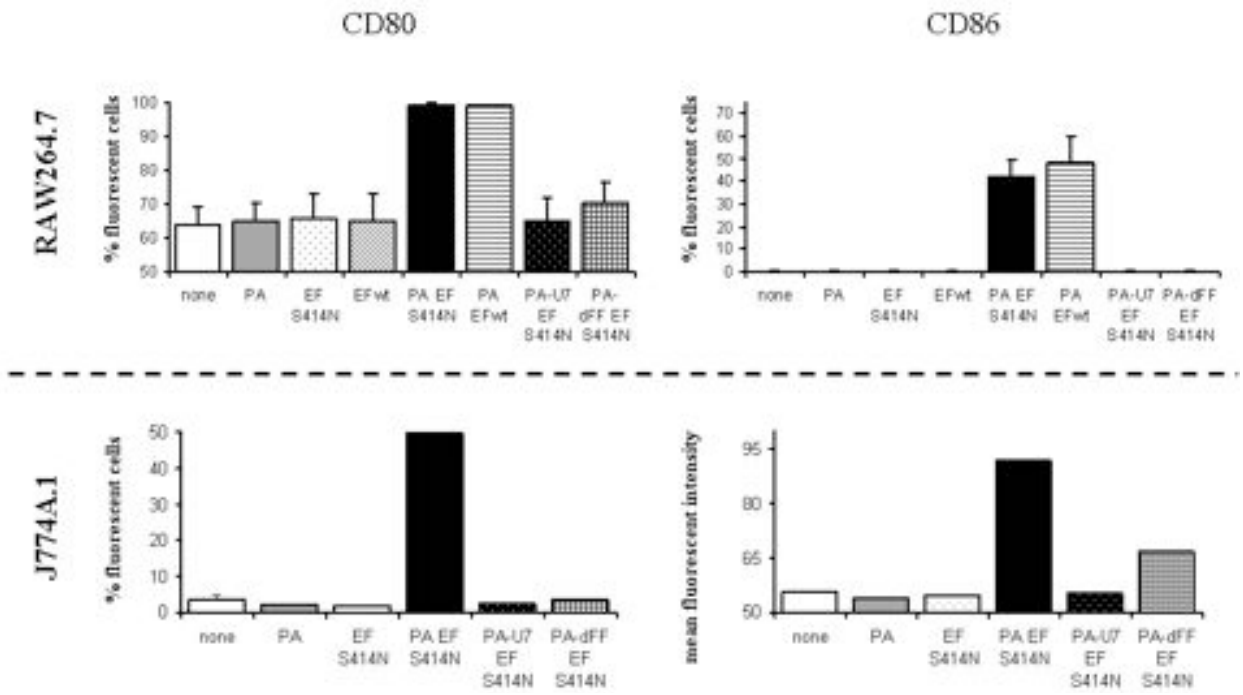

Figure 23. Costimulatory molecule expression in macrophage cell lines after treatment with EdTx derivatives.

Mouse macrophage cell lines RAW264.7 and J774A.1 were plated on a 24-well plate at 10^6 cells/ml and treated for 24 hours with the following combination of edema toxin subunits (2 µg/ml): i) PA wild-type (PA) alone (▒), ii) EF S414N alone (▒), iii) EF (▒), iv) PA + EF S414N (■), v) PA + EF (▒), vi) PA-U7 + EF S414N (■), vii) PA dFF + EF S414N (▒). The expression of costimulatory molecules CD80 and CD86 was assessed by flow cytometry. Results are from four separate experiments.

We also assessed how EdTx subunits could affect the ability of macrophages to uptake an antigen. Both RAW264.7 and J774A.1 exhibited increased ability to uptake a soluble antigen (i.e., OVA) after a 24-hour treatment with the EdTx containing PA (Figure 24). We also used an attenuated strain of *Salmonella typhimurium* expressing GFP as an example of particulate antigen and found that a treatment with EdTx enhanced the uptake of such antigen, in a greater fashion than CT, both in J774A.1 and RAW264.7 macrophages but to a lesser extent than CpG in RAW264.7 macrophages (Figure 25). Again, future studies will determine whether an EF moiety lacking enzymatic activity can modulate antigen uptake by APCs.

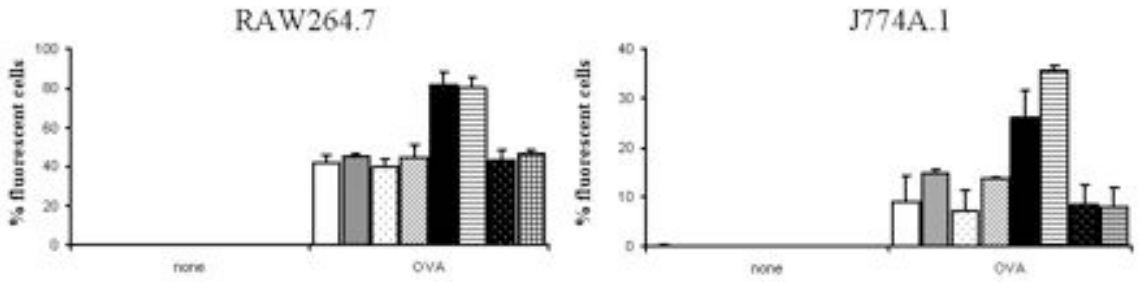

Figure 24. Ovalbumin uptake by macrophage cell lines after treatment with EdTx derivatives.

Mouse macrophage cell lines RAW264.7 and J774A.1 were plated on a 24-well plate at 10^6 cells/ml and treated for 24 hours with the following combination of edema toxin subunits (2 µg/ml): i) PA wild-type (PA) alone (▒), ii) EF S414N alone (⊡), iii) EF (▨), iv) PA + EF S414N (■), v) PA + EF (⊟), vi) PA-U7 + EF S414N (▪), vii) PA dFF + EF S414N (⊞). Medium was removed and cells were incubated for 15 min with OVA-Alexa Fluor 488 (5 µg/ml) (Molecular Probes). The uptake of ovalbumin was assessed by flow cytometry.

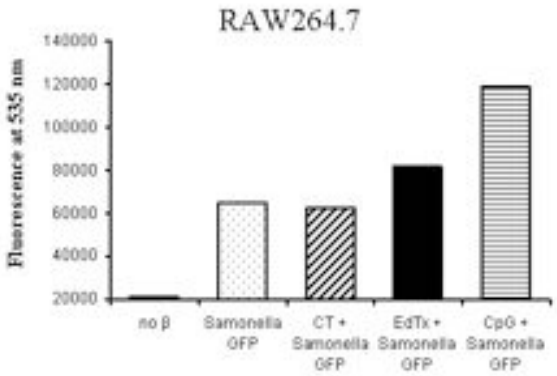

Figure 25. Salmonella uptake by macrophage cell lines after treatment with EdTx, CpG or CT.

Mouse RAW264.7macrophages were plated on a 24-well plate at 10^6 cells/ml and treated for 24 hours with PA plus EFS414N (2 µg/ml) (■). CT (1 µg/ml) (▨) and CpG 1826 (5 µg/ml) (⊟) were used as controls. Medium was removed and cells were incubated for 2 hours with a culture of *Salmonella*-GFP (OD610 ~ 0.7). The uptake of *Salmonella*-GFP was assessed by flow cytometry.

5.7.3 Role of Nerve Growth Factor in the adjuvant activity of EdTx

Our separate studies have now shown a link between the adjuvant activity of CT and another innate factor, the nerve growth factor (NGF). NGF is produced by a variety of cells including Th2 cells [174] and eosinophils [175]. It was reported to induce the growth and differentiation of B cells [176] and enhance the production of antigen-specific antibodies [177]. Evidence was recently put forth that NGF is release upon stimulation by IL-1β [178]. Since CT acts as a mucosal adjuvant via mechanisms involving IL-1 and promoting Th2 cells, we investigated whether NGF was involved in the adjuvant activity of CT. The treatment of mice with an anti-NGF Ab altered the adjuvant activity of CT (Figure 26) but also the immune response to CTb itself (Figure 27).

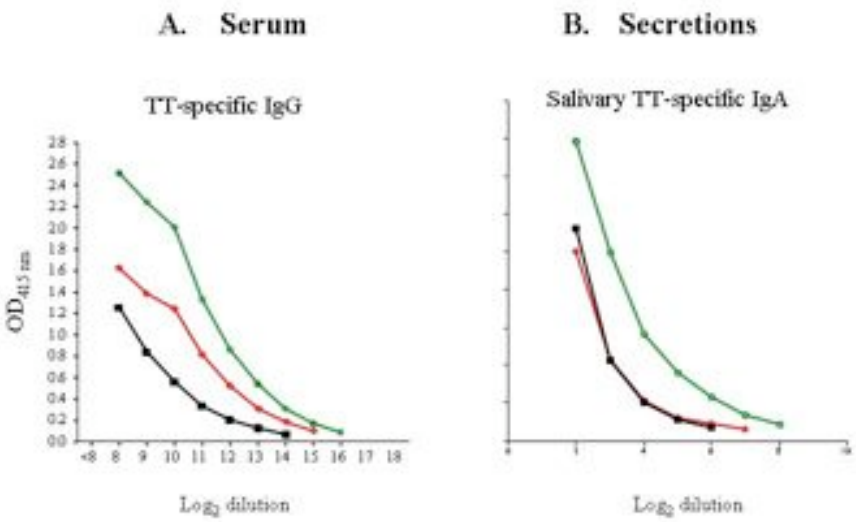

Figure 26. Anti-NGF inhibits Ag-specific responses in vivo.

Groups of mice were nasally immunized three times at weekly intervals with 20 µg of TT (tetanus toxoid) plus 0.5 µg of CT + 50 µg of anti-NGF Ab (◇), TT alone(■) or TT plus CT + 50 µg of control Ab (○). TT-specific Abs were analyzed in pool sera and saliva one week after the last immunization.

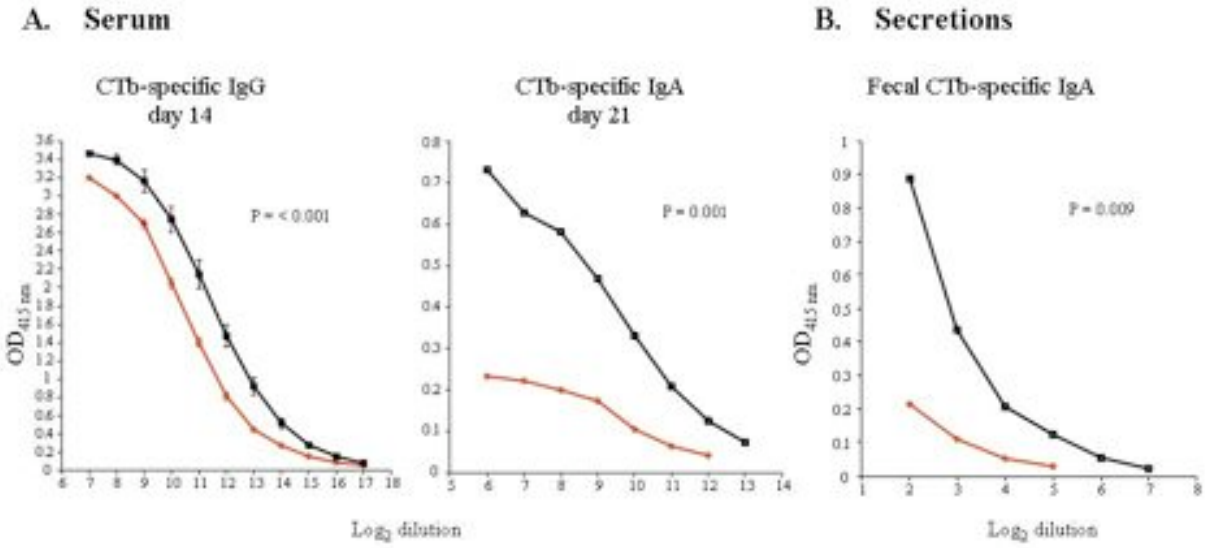

Figure 27. Anti-NGF inhibits CTb-specific responses *in vivo*.

Groups of mice were nasally immunized three times at weekly intervals with 20 µg TT + 0.5 µg CTb + control Ab (■) and 10 µg TT + 0.5 µg CTb + anti-NGF Ab (◇). TT-specific Abs were analyzed in pool sera and saliva one week after the last immunization.

The role of NGF in the adjuvanticity of CT was further demonstrated by the fact that nasal co-application of NGF 7S enhanced systemic immunity to co-administered protein vaccine and promoted mucosal SIgA (Figure 28). As previously shown for CT, NGF 7S as an adjuvant, generated Th2-related IgG subclasses.

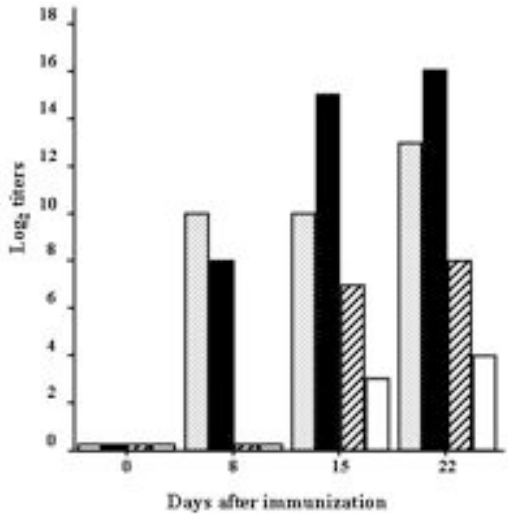

Figure 28. Nerve growth factor is a nasal adjuvant.

Groups of mice were nasally immunized three times at weekly intervals with 5 µg 7S NGF + 20 µg TT. Serum IgM (⊡), serum IgG (■), serum IgA (◪), fecal IgA (□) were analyzed in pool sera and saliva one week after the last immunization.

We next addressed whether the adjuvant activity of EdTx, which targets anthrax toxin receptors on cells, would require NGF. For these studies, we used wild-type EF with approximately five times higher enzymatic activity than the mutant EF S414N (~ 2510 versus 550 mM cAMP/min/mg protein for the mutant). Nasal treatment of mice with a polyclonal anti-NGF Ab (Chemicon International, Temecula, CA) at the time of nasal co-immunization with PA, EF and OVA, failed significantly to reduce the adjuvant activity of wild-type EdTx and subsequent immune IgG (including IgG1 and IgG2b) and IgA Ab responses to OVA in the plasma (Figure 29) and IgA Ab responses in mucosal secretions (Figure 29).

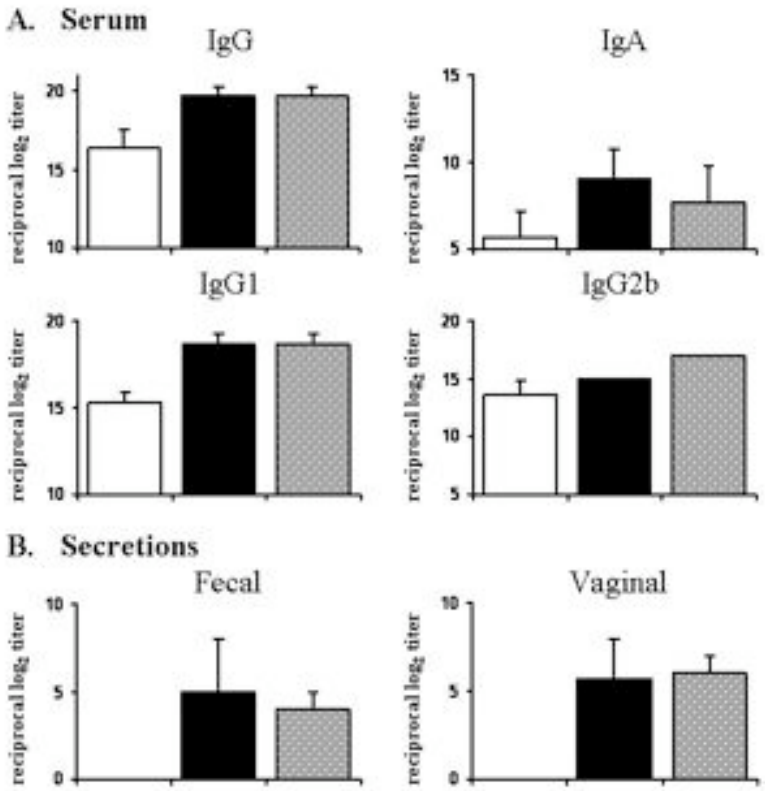

Figure 29. Anti-NGF does not inhibit nasal EdTx-induced OVA-specific responses.

Mice were nasally immunized three times at weekly intervals with 100 µg of OVA only (□), OVA plus 5 µg of PA plus 5 µg of EF S414N (■), OVA 5 µg of PA plus 5 µg of EF S414N plus 50 µg of anti-NGF Ab (▩). A. Plasma samples were collected one week after the last immunization (day 21) for OVA-specific IgA, IgG and IgG-subclass Abs. B. Fecal extracts and vaginal lavages were collected two weeks after the last immunization (day 28) to examine their content of OVA-specific IgA Ab levels. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group.

In order to confirm that NGF was not involved in the adjuvant activity of wild-type EdTx and EdTx derivatives, we examined whether co-administration of a polyclonal anti-NGF Ab would affect the immune response of wild-type EdTx. As depicted in Figure 30, EdTx enhanced anti-F1-V Ab responses above the levels achieved by nasal application of F1-V alone. Co-administration of polyclonal anti-NGF Ab at the time of nasal co-immunization with PA, EF and F1-V did not the adjuvant activity of EdTx for the co-administered antigen of *Y. pestis* (Figure 30).

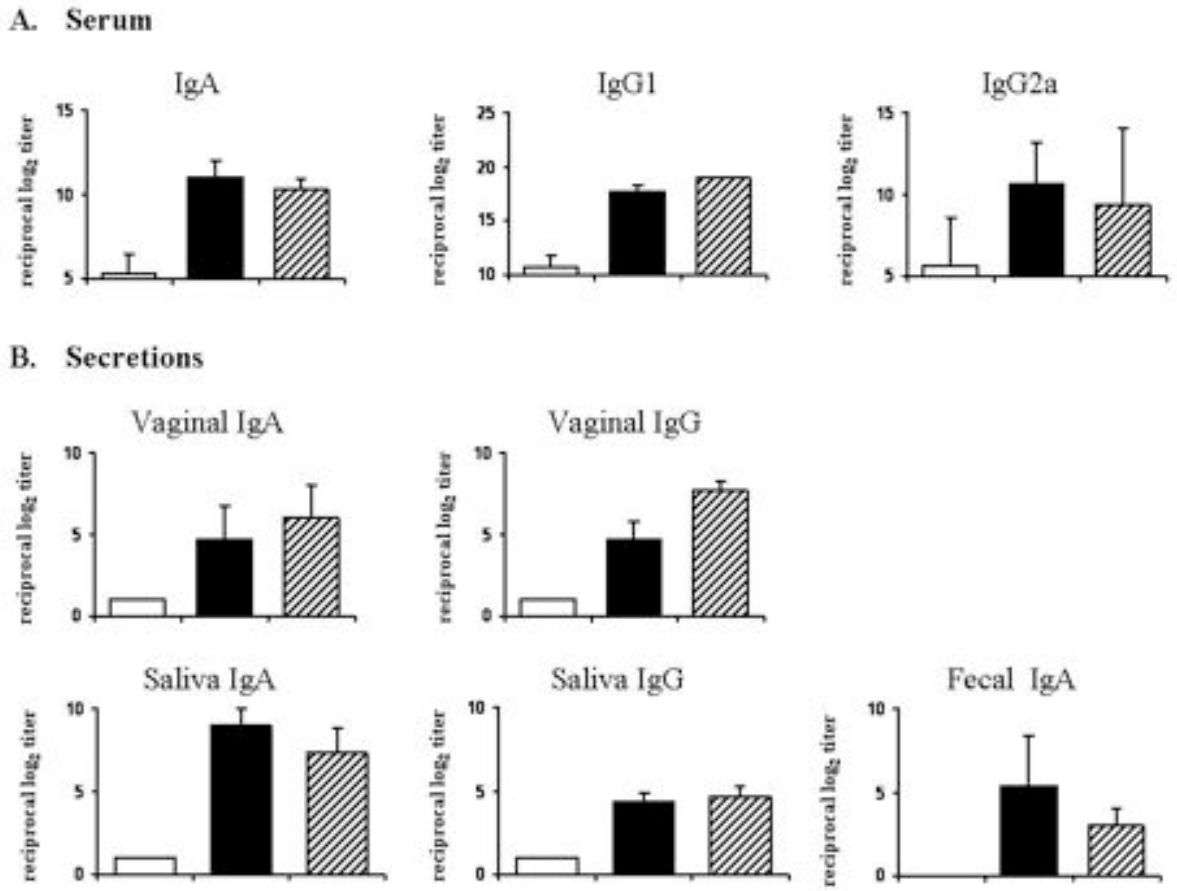

Figure 30. Anti-NGF does not inhibit nasal EdTx-induced F1-V-specific responses.

Mice were nasally immunized three times at weekly intervals with 20 µg of F1-V only (□), F1-V plus 2 µg of PA plus 2 µg of EF (■), F1-V plus 2 µg of EdTx plus 50 µg of anti-NGF Ab (◪). A. Plasma samples were collected one week after the last immunization (day 21) for F1-V-specific IgA and IgG-subclass (IgG1 and IgG2a) Abs. B. Fecal extracts, vaginal lavages and saliva were collected two weeks after the last immunization (day 28) to examine their content of F1-V-specific IgA and IgG Ab levels. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group.

The question also remained whether blocking the NGF signaling by co-administration of polyclonal anti-NGF Abs would alter the immunogenicty of EdTx iself, and more specifically, the immune response against its binding subunit PA. Data summarized in Figure 31 clearly show that anti-PA Ab responses were not affected by co-administration of EdTx and polyclonal anti-NGF Abs. In fact, these results even suggest that serum IgG1 and vaginal IgG Ab levels are increased in the groups that received this treatment.

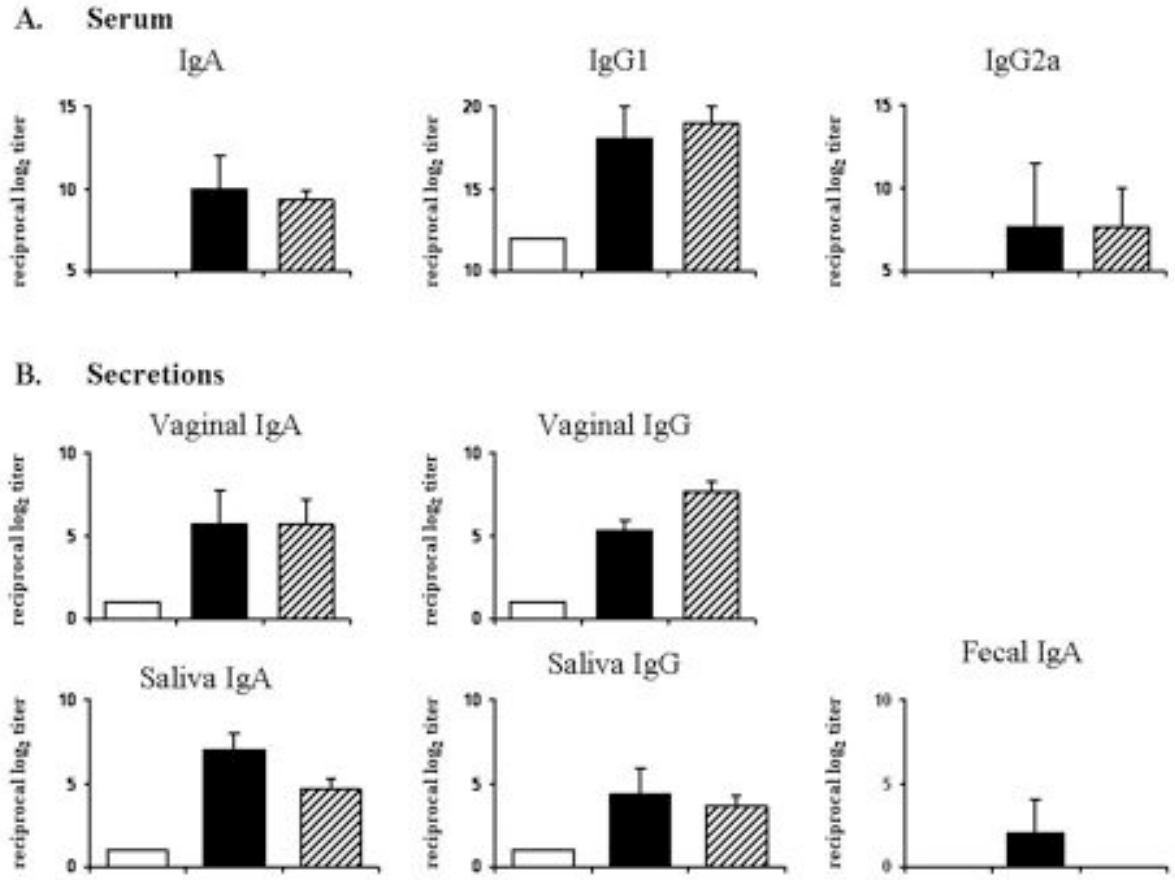

Figure 31. Anti-NGF does not inhibit nasal EdTx-induced PA-specific responses.
Mice were nasally immunized three times at weekly intervals with 20 µg of F1-V only (□), F1-V plus 2 µg of PA plus 2 µg of EF (■), F1-V plus 2 µg of EdTx plus 50 µg of anti-NGF Ab (◪). A. Plasma samples were collected one week after the last immunization (day 21) for PA-specific IgA and IgG-subclass (IgG1 and IgG2a) Abs. B. Fecal extracts, vaginal lavages and saliva were collected two weeks after the last immunization (day 28) to examine their content of PA-specific IgA and IgG Ab levels. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group.

Cholera toxin is a well-known switch factor, which induces the differentiation of IgM^+ B cells into IgA-producing cells [179]. We also found that the presence of cholera toxin in cultures of purified B cells stimulated with LPS significantly enhanced the levels of IgA secreted in culture supernatants (Figure 32). Remarkably, the addition of anti-NGF antibodies significantly reduced the enhancing effect of CT on IgA secretion (Figure 32). To date, no study has addressed

whether *Bacillus anthracis* EdTx is a switch or differentiation factor that promotes the maturation of B cells into IgA producing cells. In addition to its adjuvant activity, *in vivo*, EdTx could enhance the production of IgA antibodies by LPS-stimulated B cell cultures (Figure 32). As seen in cultures of cells treated with CT, the addition of anti-NGF Ab significantly reduced the EdTx-induced production of IgA Abs by LPS-stimulated B cells (Figure 32). The effect of anti-NGF did not seem to be a result from an overall alteration of B cell functions, since anti-NGF did not affect the EdTx-induced expression of CCR9 on LPS-stimulated B cells (data not shown).

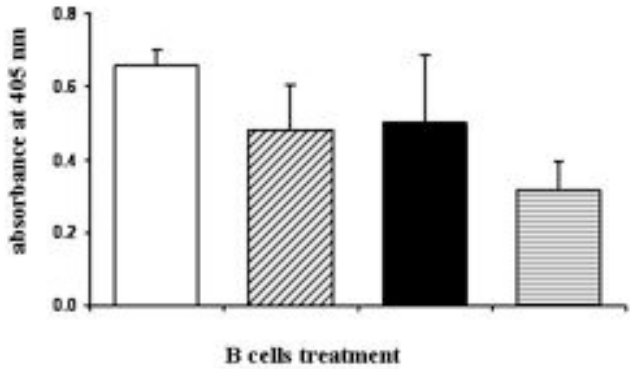

Figure 32. Anti-NGF effect on CT or EdTx-treated B cell IgA secretion.

Purified B cells fom MLN and PP a C57BL/6 mouse were incubated with LPS (10 µg/ml) alone, or LPS plus CT (0.05 µg/ml) (□), or LPS plus CT plus anti-NGF (▨), or LPS plus EdTx (0.05 µg/ml) (■), or LPS plus EdTx plus anti-NGF (▤) for three days. Supernates were collected to analyze their content of total IgA Ab.

Summary of nasal immunization studies: The nasal immunization studies above have helped determine that EdTx and a derivated with reduced enzymatic activity are nasal adjuvants capable of promoting both mucosal and systemic immunity. They also helped demonstrate that EdTx as nasal adjuvant employs distinct innate mechanisms than the ganglioside binding adjuvant cholera toxin.

5.8 Edema toxin acts as an adjuvant for serum Ab responses to topically co-applied vaccine antigens

The ganglioside-binding adjuvants CT and LT-1 were shown to enhance immunity to antigen given by transcutaneous application. In mice, CT has been applied on the skin with an antigen at concentrations ranging from 10 [111] to 100 µg [180], while much lower doses of .5 or 1 µg of native CT were optimal for induction of mucosal immunity by the nasal route [77, 171]. EdTx S414N doses of 5 µg could promote optimal immune responses to nasal vaccines [171]. Therefore, EdTx and CT were used at 25 or 50 µg for transcutaneous studies. Mice immunized by transcutaneous application of OVA with CT or EdTx (PA plus EF S414N) developed higher levels of OVA-specific IgG Abs in the serum than those transcutaneously immunized with OVA

alone (Figure 33). A the peak of the immune response (i.e., two weeks after the last cutaneous immunization), mice that received either EdTx or CT showed serum OVA-specific IgG1, and IgG2a Abs which indicate a mixed Th1 / Th2 type of immune response (Figure 33). A fully functional EF subunit (EF) had no significant effect on either the magnitude of serum IgG Abs or the quality (i.e., the pattern of IgG subclass Abs) of the immune response compared to EdTx S414N as adjuvant (Figure 33). We also examined the kinetics of serum IgE Abs after TCI with EdTx. Two weeks after the first TCI (i.e., day 14), we measured peak of IgE responses in the serum of mice transcutaneously immunized with either CT and EdTx and no significant difference was seen between these two groups (Figure 33). This result supports the involvement of a Th2 component in the immune responses induced by TCI with EdTx.

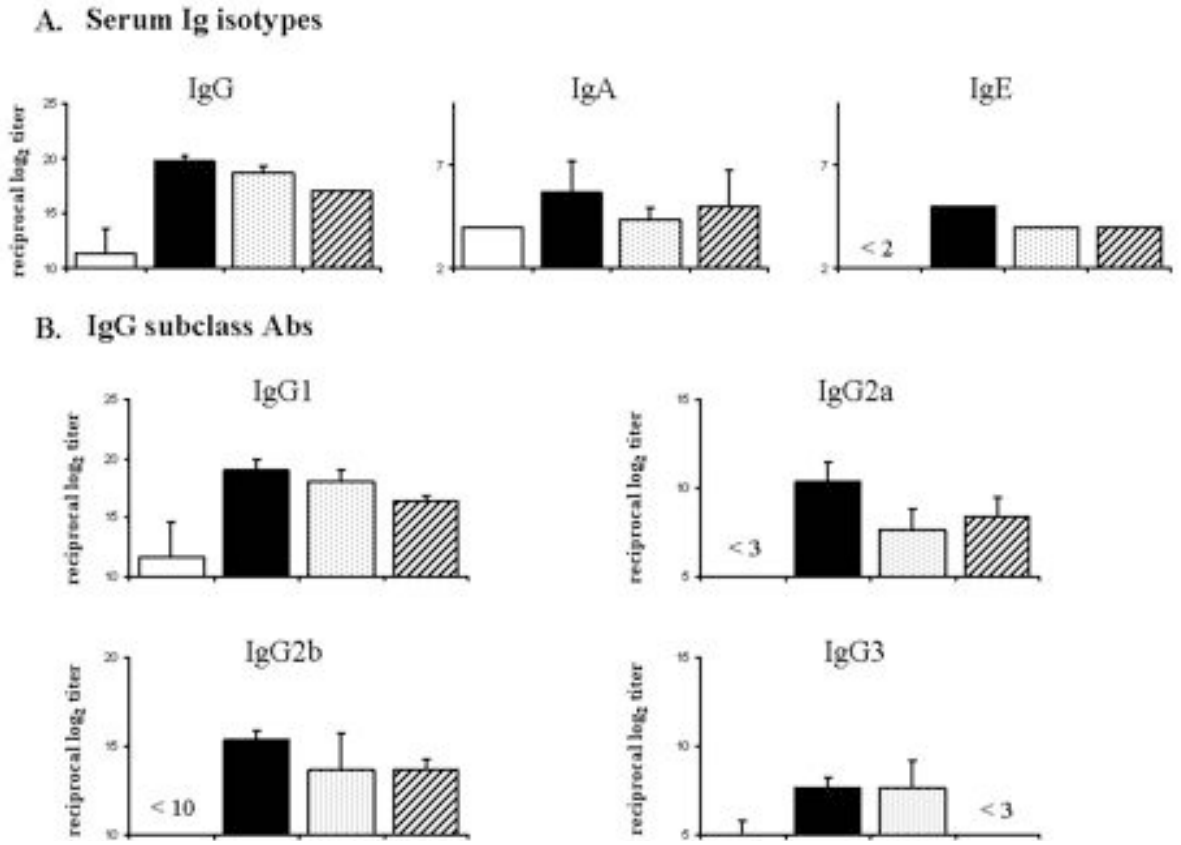

Figure 33. OVA-specific Ab responses after TCI with EdTx.

Mice were transcutaneously immunized three times at weekly intervals with 1 mg of OVA only (□), OVA plus 50 µg of PA plus 50 µg of EF S414N (■), OVA plus 50 µg of PA plus 10 µg of EF (⊡) and OVA plus 25 µg of CT (◪). A. Plasma samples were collected one week after the second immunization (day 14) to examine their content of IgE Ab levels and two weeks after the last immunization (day 28) for OVA-specific IgG and IgA Abs. B. Plasma samples were collected two weeks after the last immunization (day 28) to examine their content of OVA-specific IgG1, IgG2a, IgG2b and IgG3 Ab levels. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group.

Neither CT nor EdTx as transcutaneous adjuvant promoted any significant OVA-specific serum IgA Abs at the doses tested (Figure 33). However, high levels of CTb-specific IgA were detected in the serum (data not shown), indicating that IgA can be induced by transcutaneous immunization. This discrepancy could result from the relative immunogenicity of CTb versus OVA or the ability of each antigen to reach APCs for optimal induction of immune responses. Furthermore, the barrier provided by the outermost layer of the skin, or stratum corneum, could be an obstacle to antigen/adjuvant mixture penetration. However, treatment of the skin prior to immunization including hydration, disruption (i.e., Emery paper) or removal (by waxing) of the stratum corneum, failed to induce Ag-specific serum IgA Abs (data not shown).

5.9 Antibody responses induced by EdTx as a transcutaneous adjuvant in saliva and mucosal secretions

We next examined whether EdTx could induce antigen-specific Ab immune responses in the saliva and mucosal secretions. Fecal extracts and vaginal lavages were collected on days 14, 21 and 28. Saliva was collected on days 21 and 28. Consistent with the lack of IgA responses in the serum, no OVA-specific IgA Abs were detectable in the saliva and mucosal secretions. Nonetheless, substantial levels of OVA-specific IgG Abs were measured at day 28 in both the saliva and the vaginal lavages, collected from mice transcutaneously immunized with EdTx or CT (Figure 34).

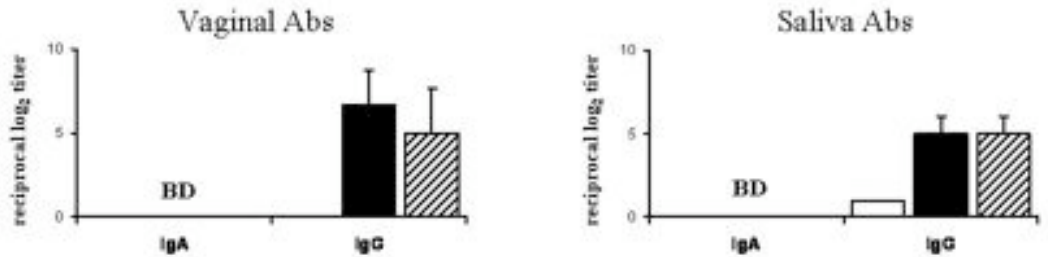

Figure 34. OVA-specific Abs in the secretions after TCI with EdTx.

Mice were transcutaneously immunized three times at weekly intervals with 1 mg of OVA only (□), OVA plus 50 µg of PA plus 50 µg of EF S414N (■) and OVA plus 25 µg of CT (▨). Vaginal lavages and saliva were collected two weeks after the last immunization (day 28) to examine their content of OVA-specific IgG and IgA. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group. BD, Below detection levels.

We also assessed whether transcutaneous EdTx could trigger a long lasting immune response by looking at serum and salivary Abs six weeks after the last immunization. As shown in Figure 35, the levels of Ab responses on day 56 in the groups that received OVA plus EdTx are similar to

those measured on day 28. Moreover, OVA-specific IgG1, IgG2a and IgG2b Abs are still present, highlighting that the nature of the immune response remains the same over time. Antibodies produced in the secretions are usually the first to disappear; however, we were able to detect substantial amounts of OVA-specific IgG Abs in the saliva (Figure 35). No OVA-specific IgA Abs were detected in the saliva or any other mucosal secretions (data not shown).

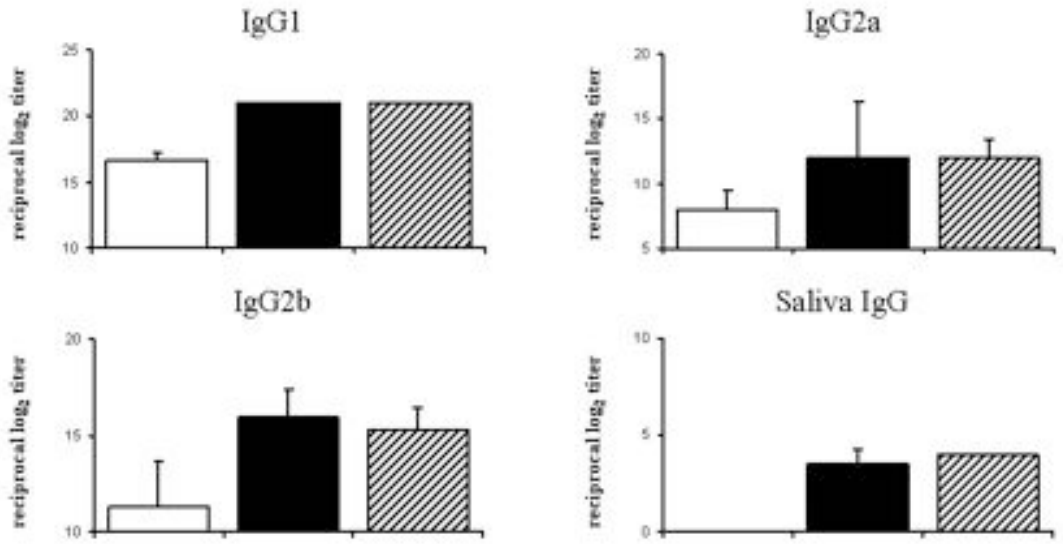

Figure 35. Long-lasting OVA-specific Abs after TCI with EdTx.

Mice were transcutaneously immunized three times at weekly intFervals with 1 mg of OVA only (□), OVA plus 50 µg of PA plus 50 µg of EF S414N (■) and OVA plus 25 µg of CT (▨). Plasma samples and saliva were collected six weeks after the last immunization (day 56) to examine their content of OVA-specific IgG, IgG2a, and IgG2b Abs. The results are expressed as the reciprocal log_2 titers ± one SD and are from 3 experiments and 4 mice/group.

5.9.1 CT and EdTx differentially affect immune cells in the GI tract after TCI application

16 hours after abdominal application of 50 µg of CT, we observed a significant accumulation of fluid in the small intestine of mice (Figure 36). The magnitude of fluid accumulation was comparable to that induced after oral immunization with 10 µg of CT. This observation supports the report by Belyakov et al., who showed that transcutaneous application of an HIV peptide Ag along with LT, induced LT-carrying APCs in the Peyer's Patches of immunized mice [6]. In contrast to CT, EdTx applied on the skin failed to induce fluid accumulation in the GI tract (data not shown). This finding suggests that EdTx and CT have different trafficking pathways after transcutaneous application. Ongoing studies in the laboratory are addressing this point and the nature of cells targeted by CT and EdTx in the epicutaneous regions and draining lymph nodes.

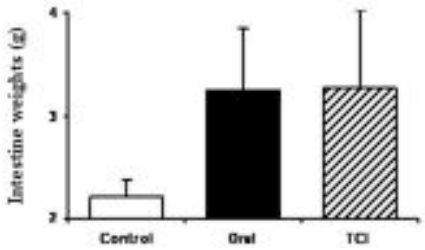

Figure 36. Intestine weights 16 hours after oral or transcutaneous administration of CT.

16 hours after topical application of CT (50 µg) or oral administration (15 µg), mice were sacrificed and weighed. The intestine were removed and weighed individually. EdTx does not induce intestinal fluid accumulation and thus, does not affect the weight of intestines.

5.10 Topical application of EdTx promotes immunity to anthrax toxin components

Our nasal immunization studies have shown that the presence of EF increases PA-specific [171] and EF-specific Abs in the plasma and the secretions of mice immunized nasally with EdTx. Similarly, co-administration of EF resulted in higher levels of plasma PA-specific IgG, as well as IgG1, IgG2b, IgG2a Abs than in mice transcutaneously immunized with the binding subunit PA alone (Figure 37). PA-specific IgG3 Abs, which were not detectable after immunization with PA only, were not induced by co-application of EF. As indicated above for OVA-specific Ab responses, we failed to detect PA-specific IgA Abs in the serum, saliva or mucosal secretions of mice exposed to EdTx by TCI. But again, saliva and vaginal lavages exhibited PA-specific IgG Abs levels, which were significantly higher after TCI with EdTx than with PA alone (Figure 37). It is worth indicating that EF-specific IgG Abs were also induced in the serum and saliva after TCI with EdTx S414N or EdTx consisting of the wild-type EF.

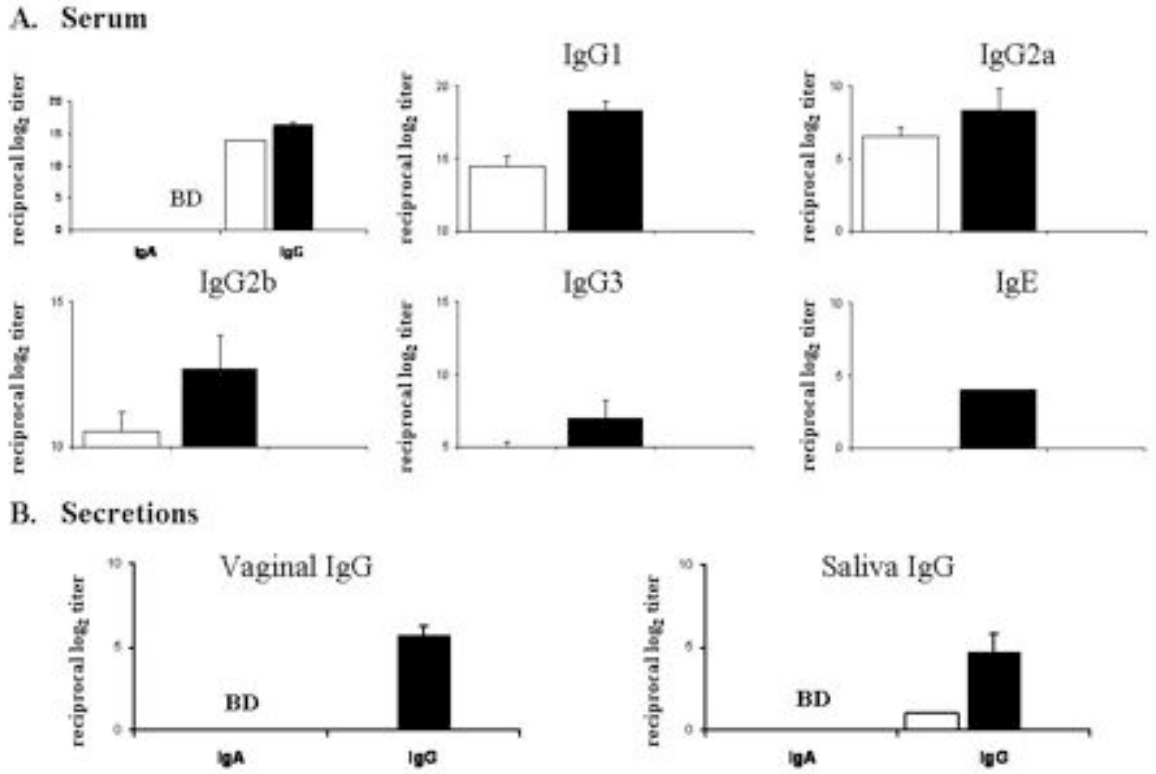

Figure 37. PA-specific Ab responses after TCI with EdTx.

A. PA-specific Ab responses were measured in the serum of mice transcutaneously immunized three times at weekly intervals with 1 mg of OVA plus 50 µg of PA (□), OVA plus 50 µg of PA plus 50 µg of EF S414N (■), and OVA plus 25 µg of CT (◪). Plasma samples were collected one week after the second immunization (day 14) and one week after the last immunization (day21) and examined for their content of OVA-specific IgE Abs, IgA, IgG and IgG-subclass Abs. B. Saliva and vaginal lavages were collected one week after the third immunization and analyzed for the detection of IgA and IgG Abs. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group. BD, Below detection levels.

One could ask whether EdTx can also induce immune responses directed against its enzymatic subunit EF. As shown in Figure 38, transcutaneous immunization with EdTx also promoted anti-EF Ab responses in the sera, which were characterized with high levels of IgG1 and very low to no IgG2a Abs. We also failed to detect EF-specific IgA Abs in either the plasma or mucosal secretions or saliva. Furthermore, no EF-specific IgG Abs was detected in saliva or vaginal washes as example of mucosal secretions.

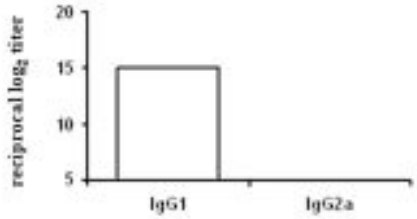

Figure 38. EF-specific Ab responses after TCI with EdTx: IgG1/ IgG2a ratio.

Mice were transcutaneously immunized three times at weekly intervals with OVA plus 50 µg of PA plus 50 µg of EF S414N. Plasma samples were collected one week after the last immunization (day 21) for the assessment of EF-specific IgG1 (□) and IgG2a (■) Abs. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group. ($p < 0.05$).

5.11 The transcutaneous adjuvant edema toxin promotes mixed Th1 and Th2 cytokine and delayed-type hypersensitivity responses

In order to examine the pattern of cytokines secreted by $CD4^+$ T cells in mice topically immunized with the adjuvant EdTx, splenocytes from immunized mice were stimulated for 5 days with OVA (1 mg/ml). As illustrated in Figure 39, T helper 2-type cytokines (i.e. IL-5, IL-6, IL-10 and IL-13) as well as IFN-γ, a Th1-type cytokine, were found in supernatants of cultured splenocytes from mice immunized with OVA plus EdTx. Likewise, the immunization OVA plus CT was characterized by the secretion of the same cytokines by restimulated splenocytes, underlining a mixed profile also. None of the cytokines tested were detected in splenocytes originating from mice given OVA plus PA (Figure 39).We further characterized topical EdTx-induced T cell responses by analyzing ear thickness of mice transcutaneously immunized with OVA and EdTx or CT, after *in vivo* challenge with OVA. While mice transcutaneously administered OVA exhibited limited delayed-type hypersensitivity (DTH) responses as measured by ear thickness, those immunized with OVA and EdTx or CT showed significantly higher DTH responses (Figure 40). Consistent with the profile of Th cytokine responses, similar levels of DTH responses were measured in mice given EdTx and CT.

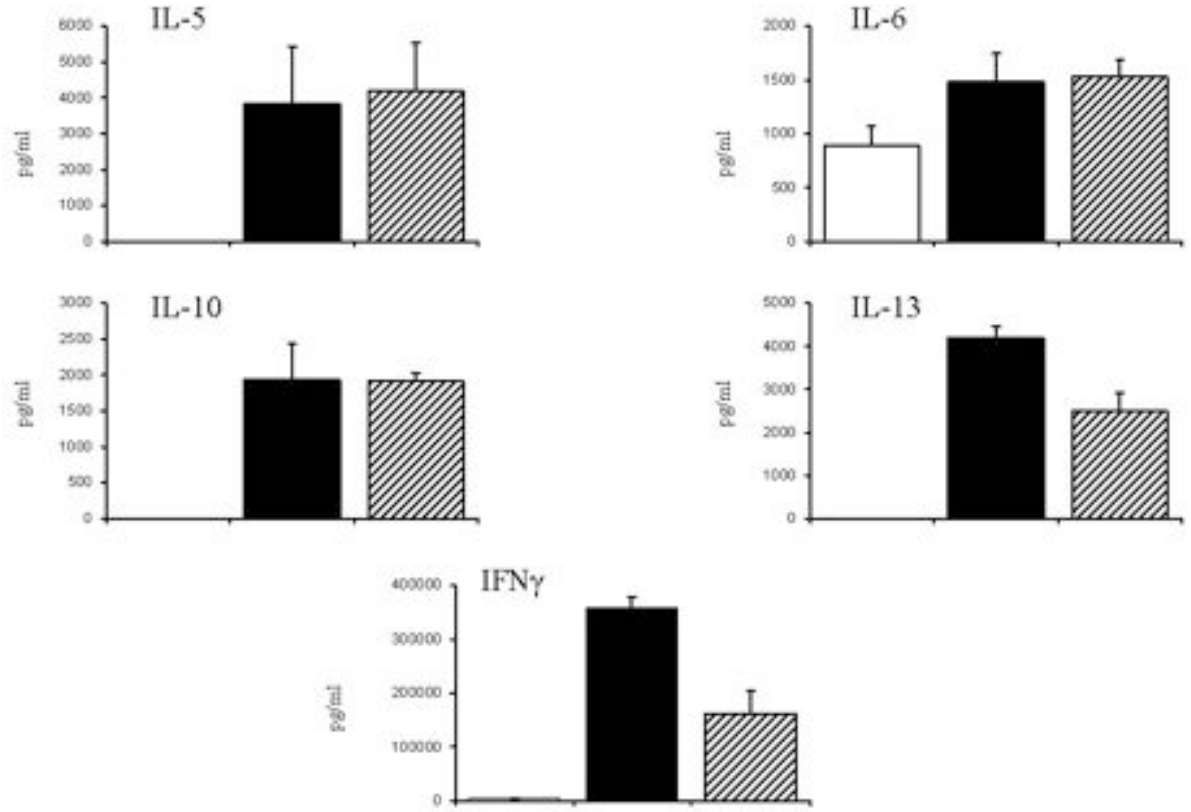

Figure 39. OVA-specific Th cell cytokine responses after TCI with EdTx.

Spleen CD4$^+$ T cells were isolated from mice transcutaneously immunized with 1 mg of OVA plus 50 µg of PA (□), OVA plus 50 µg of PA plus 50 µg of EF S414N (■), and OVA plus 25 µg of CT (▨), seven days after the boost. They were restimulated *in vitro* with OVA (1 mg/ml). After a 5-day culture, supernatants were collected and submitted to an ELISA assay to evaluate their content of the following cytokines: IL-5, IL-6, IL-10, IL-13 and IFN-γ. The results are expressed as the mean ± 1 SE and are representative of 3 separate experiments. ($p < 0.05$).

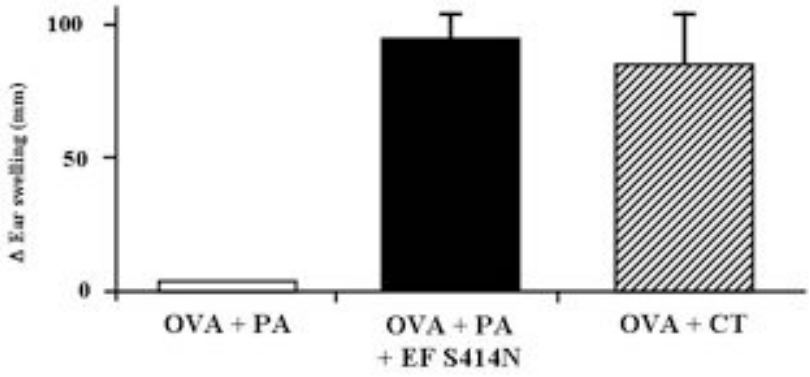

Figure 40. Delayed-Type Hypersensitivity responses after TCI with EdTx.

Mice transcutaneously immunized with OVA + PA, or OVA + EdTx (EF S414N), or OVA + CT (three times at weekly intervals) were challenged with the antigen OVA (subcutaneous injection in the ear). The DTH reaction against OVA was measured as the increase in ear thickness in µm 24 hours later as described in *Materials and Methods*. Control mice received sterile PBS. ($p < 0.05$).

5.12 Sublingual immunization with EdTx as adjuvant

The epithelium lining the buccal mucosa lacks the cornified surface (i.e., stratum corneum) that covers the skin and the keratinized regions of the oral cavity such as the gingiva and palate, (Figure 41). Consequently, the permeability of the buccal mucosa is greater than that of the skin (reviewed in Nicollazzo JA, 2005 [181]). Due to the direct drainage of blood from the buccal epithelium into the internal jugular vein, diffusion of molecules in the intestine may be avoided setting buccal immunization apart from oral immunization. Furthermore, the proximity of cervical lymph nodes could lead to stimulation of the same inductive sites after nasal and sublingual immunization.

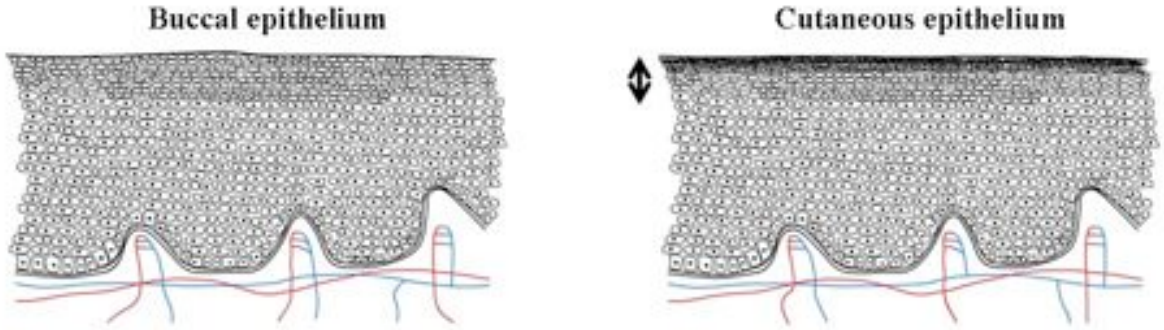

Figure 41. Structures of the skin and the buccal epithelia.

Both epithelia are stratified squamous epithelia. Top represents the buccal epithelium; bottom, the cutaneous epithelium. Arrow shows the region associated with barrier properties properties (cornified layer). [Adapted from Nicollazzo JA [181]].

The adjuvant activity of EdTx for sublingual immunization was evaluated in mice given 100 µg of *Y. pestis* F1-V antigen and 15 µg of EdTx, 3 times at weekly intervals. Sublingual application of F1-V alone did not induce significant F1-V-specific serum IgG Abs. Co-application of EdTx significantly increased F1-V-specific IgG Ab responses and promoted all IgG subclasses, as well as some IgA Abs (Figure 42). Most interestingly, EdTx as sublingual adjuvant induced F1-V-specific IgA Ab responses in the saliva and vaginal lavages, but no significant amounts in the fecal extracts. Surprisingly, while significant levels of F1-V-specific IgG Abs in the vaginal lavages and saliva were measured in mice immunized with F1-V and EdTx, none were detectable in the fecal extracts of the same mice (Figure 42). The later results argue against the induction of immunity by ingestion of EdTx and F1-V.

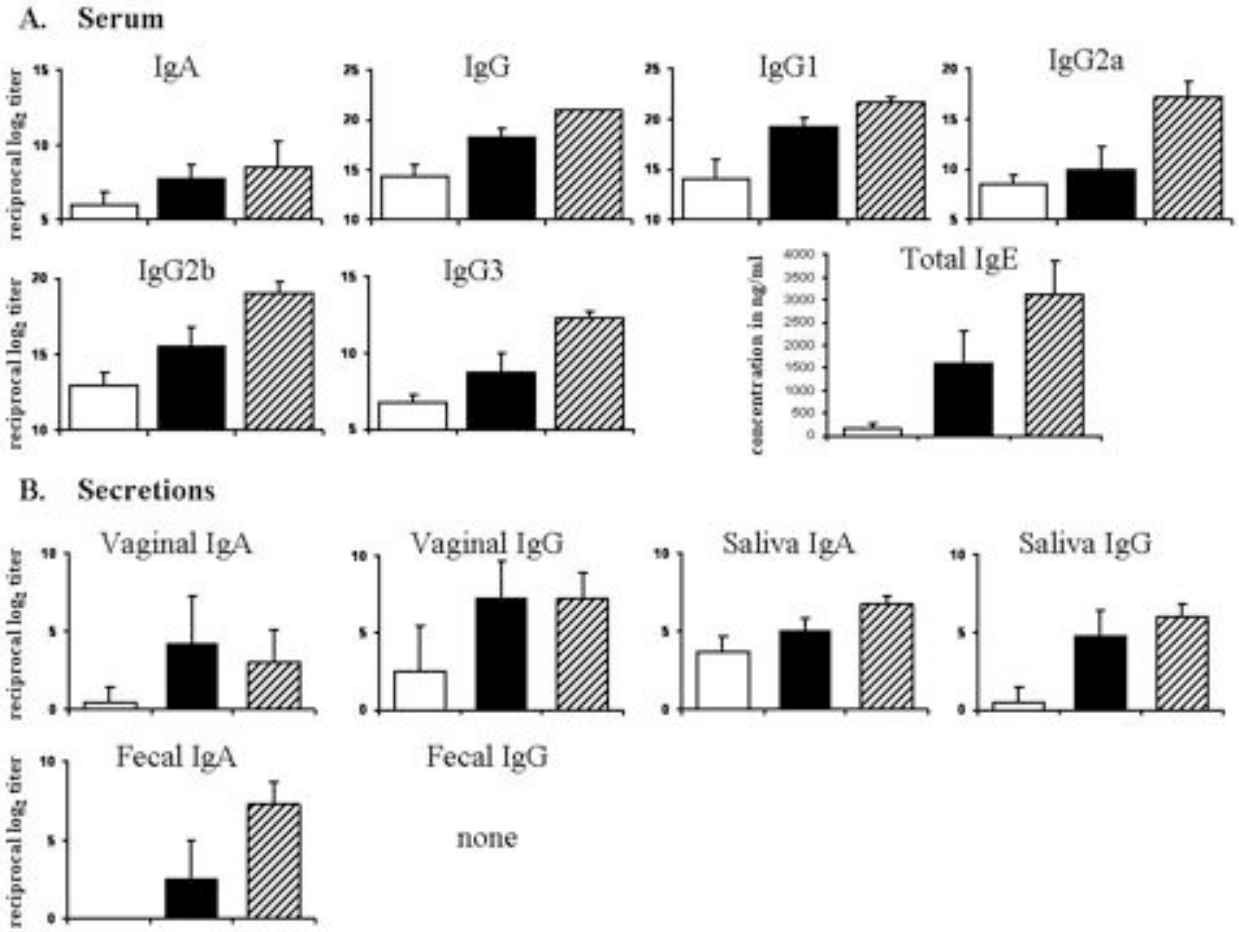

Figure 42. F1-V-specific Ab responses after sublingual immunization with EdTx.

Mice were sublingually immunized three times at weekly intervals with 100 µg of F1-V only (□), F1-V plus 15 µg of PA plus 15 µg of EF (■) and F1-V plus 3 µg of CT (◪). A. Plasma samples were collected one week after the last immunization (day 21) for the assessment of F1-V-specific IgA, IgG and IgG-subclass Abs. B. Vaginal lavages, saliva and fecal extracts were collected two weeks after the last immunization (day 28) to examine their content of

F1-V-specific IgG and IgA. The results are expressed as the reciprocal log_2 titers ± one SD and are from 3 experiments and 4 mice/group. ($p < 0.05$).

As for the other routes of immunization above, we asked whether anti-PA responses were induced after sublingual immunization with EdTx. Figure 43 shows the induction of high levels of anti-PA Abs in the serum, saliva, and vaginal washes of mice immunized via the sublingual route with EdTx. The plots in Figure 43 may let believe that some anti-PA Abs are present in mice given F1-V alone or F1-V plus CT. In fact, there was no anti-PA Abs in these groups and values shown correspond to the minimum serum dilution used in the ELISA.

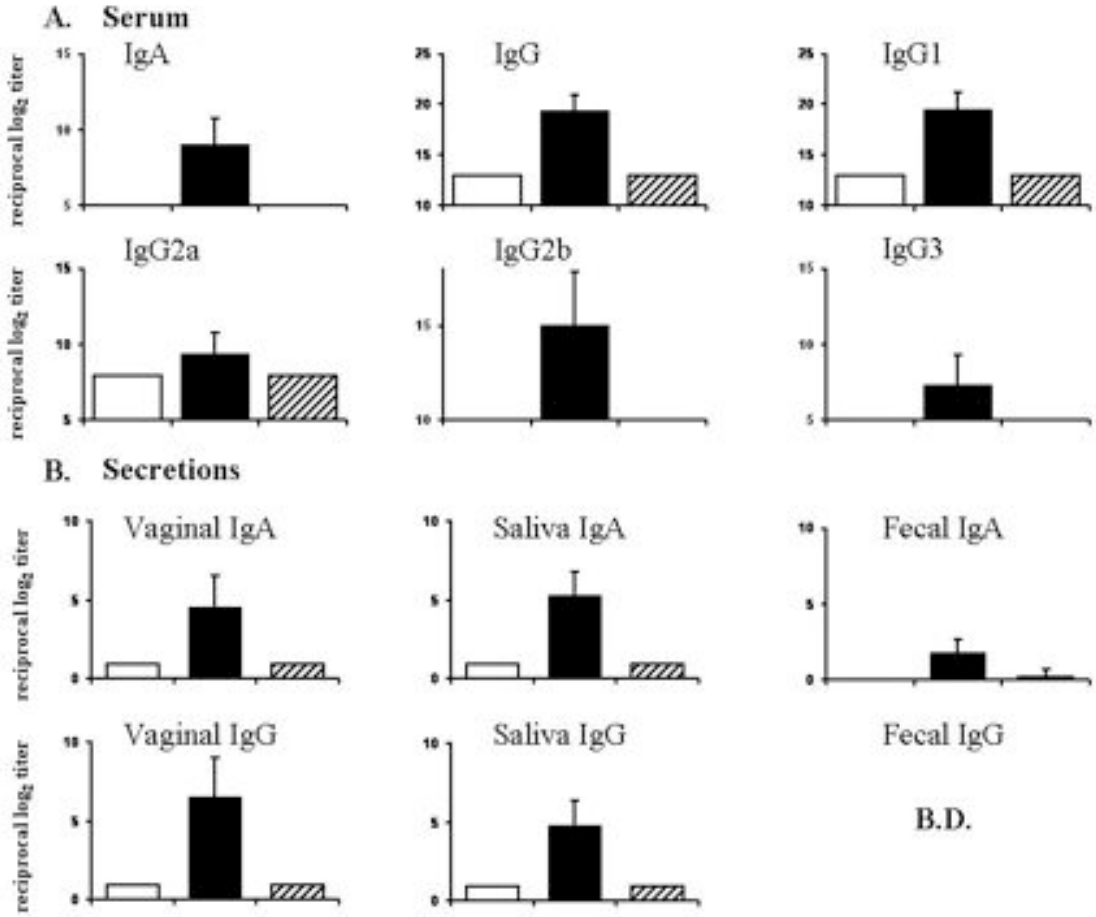

Figure 43. PA-specific Ab responses after sublingual immunization with EdTx.

Mice were sublingually immunized three times at weekly intervals with 100 µg of F1-V only (□), F1-V plus 15 µg of PA plus 15 µg of EF (■) and F1-V plus 3 µg of CT (◪). A. Plasma samples were collected one week after the last immunization (day 21) for the assessment of PA-specific IgA, IgG and IgG-subclass Abs. B. Vaginal lavages, saliva and fecal extracts were collected two weeks after the last immunization (day 28) to examine their content of PA-specific IgG and IgA. The results are expressed as the reciprocal log_2 titers ± one SD and are from 3 experiments and 4 mice/group. (Difference only between EdTx and antigen groups: $p < 0.05$).

We also confirmed that when used as an adjuvant for sublingual vaccines, EdTx could induce immune responses directed against its enzymatic subunit EF. As shown in Figure 44, transcutaneous immunization with EdTx promoted anti-EF Ab responses in the sera, which were characterized with high levels of IgG1 and low IgG2a Abs. We failed to detect EF-specific IgA Abs in either the plasma or mucosal secretions or saliva. Furthermore, no EF-specific IgG Abs were detected in the saliva or vaginal washes as example of mucosal secretions (data not shown).

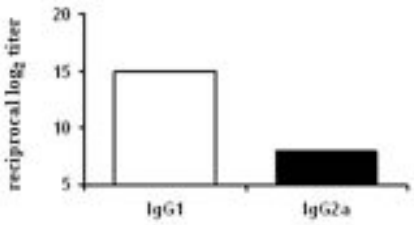

Figure 44. EF-specific Ab responses after sublingual immunization with EdTx: IgG1/ IgG2a ratio.

Mice were sublingually immunized 3 times at weekly intervals with F1-V + 15 µg of PA + 15 µg of EF. Plasma samples were collected one week after the last immunization (day 21) for the assessment of EF-specific IgG1 (□) and IgG2a (■) Abs. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from 3 experiments and 4 mice/group. ($p < 0.05$).

5.13 EdTx is an adjuvant for systemic vaccines

We have demonstrated that EdTx is a mucosal adjuvant for co-administered Ags like ovalbumin or F1-V fusion protein from *Y. pestis*, and displays adjuvant activity via various routes of immunization (i.e. nasal, transcutaneous and sublingual). We wanted to check whether EdTx adjuvanticity was only initiated at mucosal sites or if it could also be established systemically. For that purpose, we immunized C57BL/6 (I-A^b) mice i.p. with 100 µg of OVA plus EdTx. As compared with control mice that received OVA only, mice immunized with EdTx as an adjuvant showed elevated levels of OVA-specific IgG subclass Abs in the serum (Figure 45).

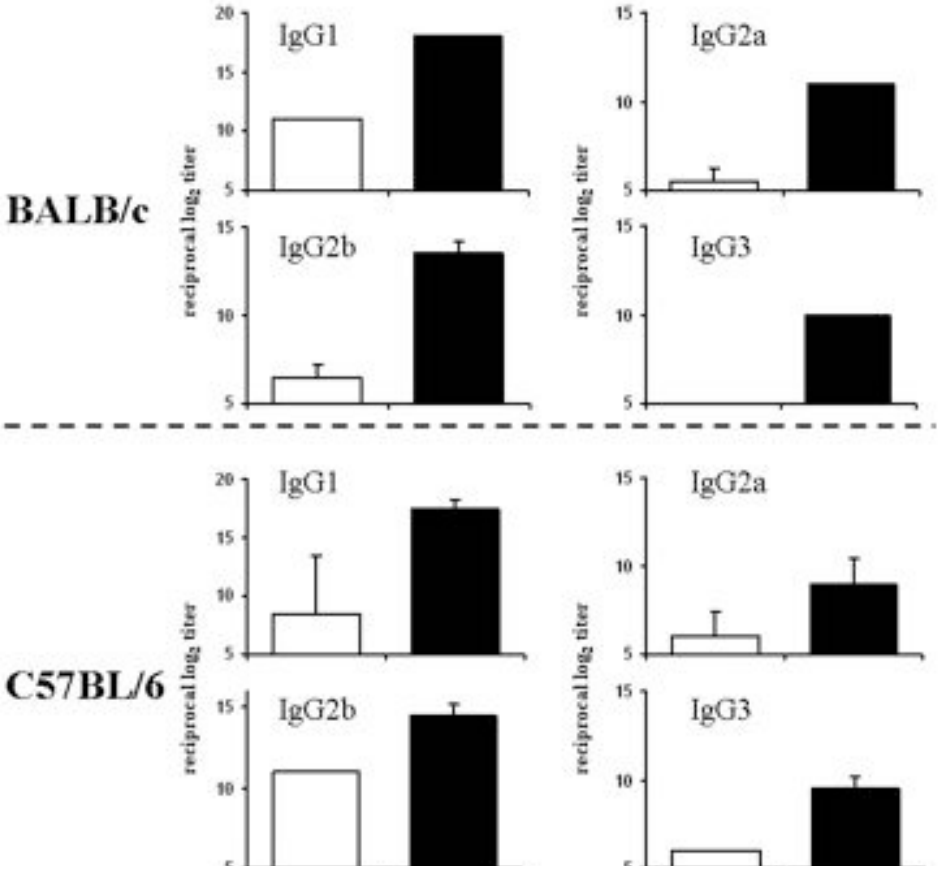

Figure 45. I.p. injection of OVA with EdTx in BALB/c and C57BL/6 mice.

C57BL/6 and BALB/c mice received an intraperitoneal injection twice a week apart consisting of 100 µg of OVA with or without 5 µg PA plus 5 µg EF S414N.

Plasma samples were collected one week after the last immunization for the assessment of OVA-specific IgG-subclass Abs. ($p < 0.05$).

The presence of IgG1 and IgG2a Abs indicated a mixed Th1- / Th2-type response, as seen after nasal immunization (Figure 45). In order to confirm that EdTx adjuvanticity was not restricted to one particular strain of mice, we extended the intraperitoneal immunizations to BALB/c mice (I-A^d) using the same protocol as the one described for C57BL/6 mice. As shown in Figure 45, EdTx enhanced OVA-specific IgG-subclass Abs in BALB/c mice. The quality of the response was similar to the one observed in i.p. immunized C57BL/6 mice with increased OVA-specific IgG1 and IgG2a Abs.

Discussion

6 Discussion

The ganglioside-binding toxins CT and LT-I are proven to be powerful adjuvants capable of enhancing immune responses to antigens co-delivered through non-invasive routes. Indeed, both toxins are well established adjuvants for vaccine antigens given by the oral, nasal, rectal, and even the transcutaneous route. The enzymatic activity of these molecules, as well as their affinity for cell-surface receptors that allow their uptake by epithelial cells of the GI tract and the olfactory nerves, are associated with adverse effects, which preclude their use in humans. We have shown that derivatives of the non-GM-binding edema toxin of *Bacillus anthracis* provide: 1) an alternative to CT and LT-I as adjuvants for non-invasive vaccines, 2) a mean to induce high and broad immunity against *Bacillus anthracis* toxins, and 3) a system to dissect the induction of mucosal IgA Abs by vaccines given by the nasal, transcutaneous and sublingual routes. This later point takes advantage of the differential induction of mucosal IgA Abs by CT and EdTx as transcutaneous adjuvants, to identify key pathways required for the induction of IgA Ab responses.

6.1 The ATR-binding EdTx is an adjuvant for non-invasive vaccines

Our most important finding was that EdTx act as an adjuvant for vaccine antigen given via multiple non-invasive routes. Nasal immunization has been a preferred route for the evaluation of adjuvant and vaccine formulation in animal models. Immune responses to nasal vaccines are believed to be initiated in the NALT, which shares similarities with intestinal Peyer's patches. Nasal application of vaccines can also lead to their diffusion to other sites of the respiratory tract, including the lungs were they can access the blood stream. For this reason, strict immunization protocols are required for nasal application of vaccine to be considered as purely mucosal immunization. Therefore, it is important that EdTx also exhibited adjuvant activity when given by the transcutaneous and sublingual routes. In this regard, skin patches are being increasingly used for delivery of drugs. Furthermore, there is a growing interest in transcutaneous vaccination after an increasing number of reports have demonstrated that mucosal IgA Abs can be induced by transcutaneous application of CT or LT-I as adjuvants [6, 14]. Nevertheless, the reliability of the results of transcutaneous immunization could be questioned because of lack of standardized method for preparing the stratum corneum before application of vaccines. Of course, the later

point is not an issue with sublingual immunization since the epithelium lining the buccal mucosa is devoid of the keratinized layer that covers the skin (Figure 41).

EdTx derivatives represent a new class of adjuvants that, in general, are as effective at inducing systemic immunity than the classical mucosal adjuvant CT. The main difference between CT and EdTx resides in the inability of EdTx to induce IgA Abs when given by transcutaneous application. Advantage could be taken of this relative weakness of EdTx and more detailed comparative studies with CT could help elucidate the signaling required for the induction of IgA Abs. Another potential benefit of EdTx derivatives as adjuvant would be their ability to provide immunity against multiple components of anthrax toxins and other co-administered vaccine antigens. In this regard, the ability of EdTx to promote immunity against *Y. pestis* F1-V indicates that grounds exist for the development of EdTx-based multivalent vaccines against multiple infectious agents.

Effort was made throughout this work to mesure IgA Abs in saliva and mucosal secretion. Potential benefits of SIgA Abs for protection of mucosal surface have been presented in the introduction. However, it is worth indicating that we did not demonstrate the presence of secretory components on these IgA Abs and therefore, we are lacking formal evidence that IgA Abs induced by EdTx in mucosal secretions are SIgA. This is especially important for saliva and vaginal secretions where serum Abs can diffuse. Nonetheless, serum levels of IgG are much higher than serum IgA Ab levels. Therefore, the fact that we found same levels of antigen-specific IgG and IgA Abs in these locations or even higher levels of IgA than IgG Abs strongly support the notion that antigen-specific IgA Abs we detected are in fact SIgA Abs.

6.1.1 Requirement for enzymatic activity of EF

The adjuvant activity of EdTx does not seem to require a fully enzymatically active molecule EF since wild-type EdTx and EdTx S414N promoted the same pattern of serum Ab responses after nasal or transcutaneous application. However, our nasal immunization studies with EF K346R suggest that a minimum of enzymatic activity is required for sustained immune responses. Most transcutaneous studies with CT were performed with the fully active molecule and the limited disclosures of studies with mutant of CT suggest the enzymatic activity is required for CT to act as an adjuvant via this route (Pascual DW, unpublished information). However, mutants of LT-1 with no or low residual enzymatic were reported to be adjuvants for antigens applied onto the skin [182].

6.1.2 Potential adverse effects

Cases of Bell's palsy (facial paresis) were reported in a clinical trial conducted in Switzerland where native LT-I was used as the adjuvant in the Berna inactivated intranasal vaccine [183]. While a direct role of LT remains to be demonstrated, CT was found to accumulate in the ON/E after nasal delivery to mice and this trafficking was GM1-dependent [184]. The B cell-binding CTA1-DD does not accumulate in CNS tissues after nasal delivery. In addition, no inflammatory responses were reported after nasal application, despite the presence of a fully active CTA1 subunit [185]. The lack of inflammatory responses in our nasal immunization studies with PA plus EF is consistent with the concept that the ganglioside-binding is a more important safety concern than the ability to increase cAMP.

One study has demonstrated that the anthrax toxin receptor ATR1 / TEM8 is expressed on epithelial cells lining the small intestine, the respiratory tissue in the lung and on keratinocytes of the skin [186]. More studies are needed to examine whether or not the anthrax toxin receptor ATR2 / GMG2 is also expressed on these sites and if other mechanisms interfere with the uptake of PA by ON/E. No adverse effect (i.e., edema or inflammation) was recorded in mice that received EdTx by transcutaneous or sublingual application at the doses used in our experiments.

6.2 Quality of immune responses induced by EdTx as adjuvant for non-invasive vaccines

Ultimately, the level of protection against infection is the most reliable metric of the quality of immune responses induced by vaccines. For ethical reasons, humans cannot be voluntarily infected to determine their level of protection. There is also growing pressure to limit animal studies. Therefore, it is important to identify correlates of protection, which can be evaluated *in vitro* from blood or mucosal secretion samples.

6.2.1 EdTx induces Abs that neutralize anthrax toxin *in vitro*

While most of our studies investigated the adjuvant activity of EdTx for unrelated protein antigens, we made the significant observation that nasal co-administration of PA and EF S414N (or wild-type EF) induces anti-PA Abs with neutralizing activity in the serum and the saliva. We

did not address the relative contribution of IgG and IgA Abs in the LeTx neutralizing activities measured in serum and saliva samples. From a vaccine development standpoint, it is encouraging that PA-specific IgG Abs were also induced in the saliva by EdTx given by the transcutaneous and sublingual routes. However, the protection provided by these antibodies will need to be established by challenge studies with anthrax toxin (i.e., LeTx) *in vivo*. The use of *Y. pestis* F1-V antigen also allows challenge studies with *Y. pestis* strains *in vivo*. In this regard, an *in vivo* challenge system is being developed with an attenuated *Y. pestis (pgm-),* in collaboration with Dr. Matthew L. Nilles (University of North Dakota).

6.2.2 T helper cytokine and DTH responses

Cell-mediated immunity and cytotoxic T cell (CTL) responses are required for protection against infection by intracellular bacteria and viruses. It is also well-accepted that Th1 cells support the CTLs. Nasal immunization studies with CT, LT-I and chimera of CT and LT-I have suggested that the binding subunits of these enterotoxins play a key role in the Th1 or Th2 polarization of the immune response [76, 77]. In this regard, the Th2 polarization of CT-induced responses is believed to result from its binding to GM1-gangliosides. On the other hand, LT-I, which binds GM1-gangliosides like CT, but also other gangliosides as well as glycoproteins, promotes mixed Th1 and Th2 responses. Interestingly, CTA1-DD, which binds to Ig receptors on B cells, promotes a balanced Th1/Th2 response. The levels of expression of ATR1 and ATR2 in the sites we tested EdTx as adjuvant, remain to be elucidated. However, regardless of the nasal, transcutaneous or sublingual route of administration, or the presence of an enzymatically active EF molecule, EdTx consistently promoted Th1 and Th2 responses.

As mentioned earlier, the functional significance of Th1 and Th2 cytokine responses was not examined after nasal immunization, but only after transcutaneous immunization with EdTx, by means of an *in vivo* challenge with the protein antigen OVA to assess a DTH response. However, an ideal way to evaluate the protective effect of the polarized Th1 / Th2 immune response observed after administration of EdTx would be to develop a model of infection with an intracellular pathogen to measure the ability of the Th1 arm of the immune response to defend the host.

6.2.3 Mucosal IgA Ab responses

Mucosal IgA Abs could be induced by nasal and sublingual applications of EdTx but not by transcutaneous application. The exact mechanisms underlying these differences remain elusive. It is likely that keratinocytes do produce a key factor for the induction of IgA. In this regard, dendritic cells of the GI tract were shown to produce retinoic acid to help differentiation of B cells into IgA producing cells. The NALT, which resembles the GALT, may contain DCs that produce retinoic acid. The μ to α isotype switch is mediated by TGF-β along with IL-4 and IL-10, as well as interactions between CD40 on B cells and its ligand on T cells [20]. The T cell-dependent mechanism for induction of IgA Abs can be further enhanced by a crosstalk between intestinal epithelial cells (IEC) and dendritic cells [29]. Furthermore, T cell-independent (TI) pathways for induction of IgA responses to intestinal flora have been described. In mice, these TI responses involve B1 cells derived from the peritoneal cavity. The mechanism relies on B-lymphocyte stimulator/B-cell-activating factor of the TNF family (BLyS/BAFF) and a proliferation-inducing ligand (APRIL), factors secreted by activated antigen-presenting cells (APC) [29]. It remains unclear whether (or how) some of these cascades are initiated after transcutaneous application of EdTx versus CT.

6.3 Mechanisms of adjuvanticity of EdTx for non-invasive vaccines

6.3.1 Contribution of innate immune factors

CT was shown to induce IL-1 production in APCs and isotype switch in B cells from μ to γ or α [187, 188]. Intestinal epithelial cells also secrete great amounts of IL-6 in the presence of CT, especially in a proinflammatory environment (IL-1, TNF-α) [189]. In addition, IL-1 and IL-6 are potent adjuvants themselves when administered nasally, and IL-1 can induce mucosal IgA Abs. Our nasal immunization studies have established that IL-1 and IL-6 are unlikely mediators of the adjuvant activity of EdTx. Along with triggering proinflammatory cytokines, CT is thought to act as an adjuvant by increasing MHC class II and costimulatory molecule expression [78, 79, 80, 188]. EdTx sharply enhanced the expression of MHC and costimulatory molecules. We also found that EdTx increased antigen uptake by macrophages, as previous reported after treatment of macrophages with CpG [190].

Nerve growth factor is known to stimulate neuron survival and maintenance. In addition, it can activate plasminogen and classical complement pathway [191, 192], accelerate wound healing [193],

chemoattract polymorphonuclear cells [192]. It was shown to be produced by effector cells such as Th2 cells, mast cells, and eosinophils in asthma and allergies [194]. This work is the first to reveal the involvement of NGF in the induction of IgA by EdTx and CT. Interestingly, the addition of an antibody to NGF to nasal vaccines only modestly affected EdTx-induced responses, while it significantly reduced the adjuvant effect of nasal administered CT, especially mucosa IgA responses. These results provide another point of difference between CT and EdTx and suggest a lower level of involvement of NGF in the adjuvant activity of EdTx when compared to CT.

6.3.2 Imprinting of homing properties on immunocytes

To date homing receptors involved in the homing of effector after nasal immunization are only partially understood. In this regard, the mechanisms underlying the induction of SIgA in the genital following nasal immunization remain to be elucidated. Our studies were not intented to unveil new homing receptors-addressins signaling for the homing of cells in the nasal-genital tract axis. However, we have clearly demonstrated that following nasal immunization with the ATR-binding edema toxin derivatives promote SIgA responses in the nasal and genito-urinary tracts like the ganglioside-binding CT. Our findings strongly suggest that the cellular receptors of the adjuvants do not play a crucial role on the acquision of homing capabilities of immune effector B and T cells initiated via stimulation of the NALT.

In contrast with homing of effector cells in the airways and genitourinary tracts, the receptors-addressins signaling for homing of immune cells to the GI tract are better described (see Figure 2). There is also evidence that an immune response can be induced in the gut after cutaneous application of CT as an adjuvant [6]. In our studies we observed the accumulation of fluid in the intestines of mice that received a cuteanous application of CT on the abdomen either alone or together with a vaccine antigen. While this observation supports the concept that gut homing occur after transcutenous immunization, we failed to detect IgA Ab response in association with this putative homing of immune cells to gut lymphoid tissues. As described in the Introduction, two major homing receptors are believed to regulate the trafficking of effector B and T cells to the small intestine: the integrin α4β7 and the chemokine receptor CCR9. Thus, it would be interesting to analyze the expression of α4β7 and CCR9 on cells isolated from gut inductive sites after TCI with CT or EdTx. Indeed, one possibility is that CT, but not EdTx, applied transcutaneously instructs skin-resident DCs (dermal DCs /andor LCs) to migrate to local skin-draining lymph nodes and imprint gut homing properties to T cells, which would most propably

translate into an increase in the expression of α4β7 and CCR9. However, these gut-migrating T cells may not provide the necessary cytokine for immunoglobulin switch by PP or MLN B cells and subsequent IgA Ab response in the intestine. Therefore, it will be iomportnat to carefully dissect the cytokine production by Ag-specific T helper cells isolated from MLN or PP to determine the nature of cellular immune response initiated at those sites after TCI. The possibility also exists that skin-resident DCs may home themselves all the way to gut inductive sites to stimulate resident T and B cells, as suggested by Belyakov et al [6]. One can assume that EdTx does not prepare skin-resident DCs to relay gut-homing instructions since we failed tp observe intestinal fluid accumulation after TCI with EdTx as adjuvant. How CT and EdTx differentially educate/ inform DCs is not understood at this time. Of course it is likely that different cascade of events are induced after binding of these molecules to their receptors after TCI. For example, factors such vitamin D3 may contribute these diferences. In fact, vitamin D3 has been shown to suppress α4β7 and CCR9 on T cells (via DCs) in the skin and thus impair gut tropism [16]. Therefore, it is possible that EdTx, but not CT, induces vitamin D3 or other factors with similar down stream effects. In any cases, our transcutaneous immunization studies once more highlighted the functional disparities between EdTx and CT as adjuvants.

Yamanaka et al [195] have demonstrated that a treatment with retinoic acid (RA) could increase the expression of α4β7 and reduce L-selectin expression on human T cells. One way to explore the homing properties of EdTx as transcutaneous adjuvant could be to adjunct RA to the vaccine preparation when applying it to the skin. RA could be one missing link/factor reposible for the initiation of gut migration after TCI with EdTx and this point will be addressed in future studies.

General summary and conclusion

7 General summary and conclusion

We have shown that derivatives of the non GM-binding edema toxin of *Bacillus anthracis* provide an alternative to CT and LT-I as adjuvant for non-invasive vaccines given by the nasal, transcutaneous or sublingual routes. As summarized in Figure 46 and Figure 47, the adjuvant activity of EdTx often results in the induction of both systemic and mucosal immunity with IgA Abs responses in mucosal secretions and saliva. Furthermore, EdTx detrivatives trigger mixed Th1 and Th2-type of immune responses. A major finding from this work is the evidence that, after nasal delivery, EdTx derivatives do not target CNS tissues as represented in Figure 46. Thus, EdTx derivatives are potential safer alternatives to ganglioside-binding molecules as adjuvant for nasal vaccines.

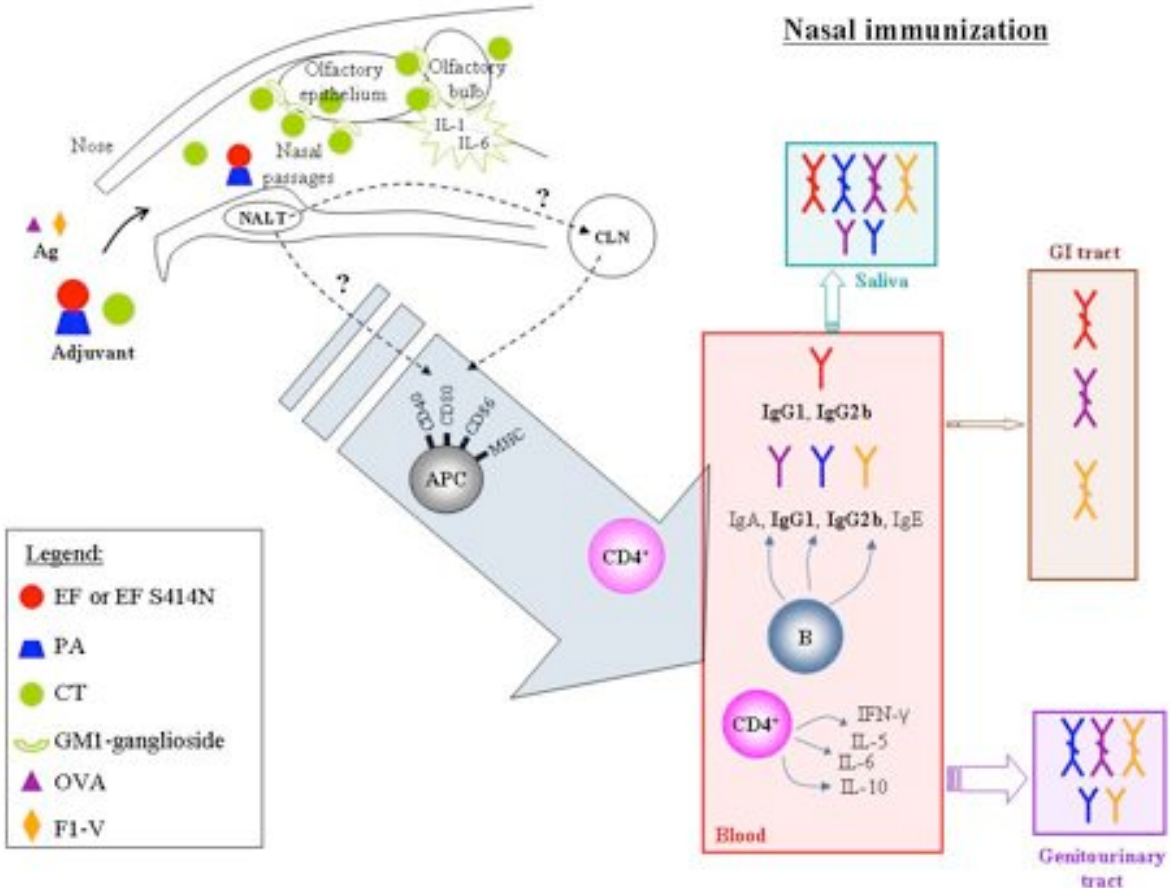

Figure 46. Schematic representations of immune responses after nasal immunizations.

Induction of Ag- or adjuvant-specific Ab responses after nasal immunization with Ag (i.e. OVA or F1-V) plus EdTx are represented. Colors of Abs match the specificity to Ag or adjuvant subunit. Mucosal IgA Abs are represented as dimers with the SC (Y); other mucosal Abs are IgG Abs represented as monomers. CT-specific Abs or Ag-specific responses following CT immunization are not represented. Nasal administration of CT leads to accumulation of CT in brain tissues (olfactory bulb and epithelium), after binding to ubiquitous gangliosides receptors, and to inflammation (represented with the secretion of IL-1, IL-6).

Contrary to sublingual and nasal immunizations, transcutaneous application of EdTx fails to promote SIgA Abs (Figure 47). Most likely, this route of immunization involves previously unknown pathways that enhance Ab responses and could thus serve as a model to study the different factors and signalings involved in the induction of IgA Abs by EdTx as compared to CT.

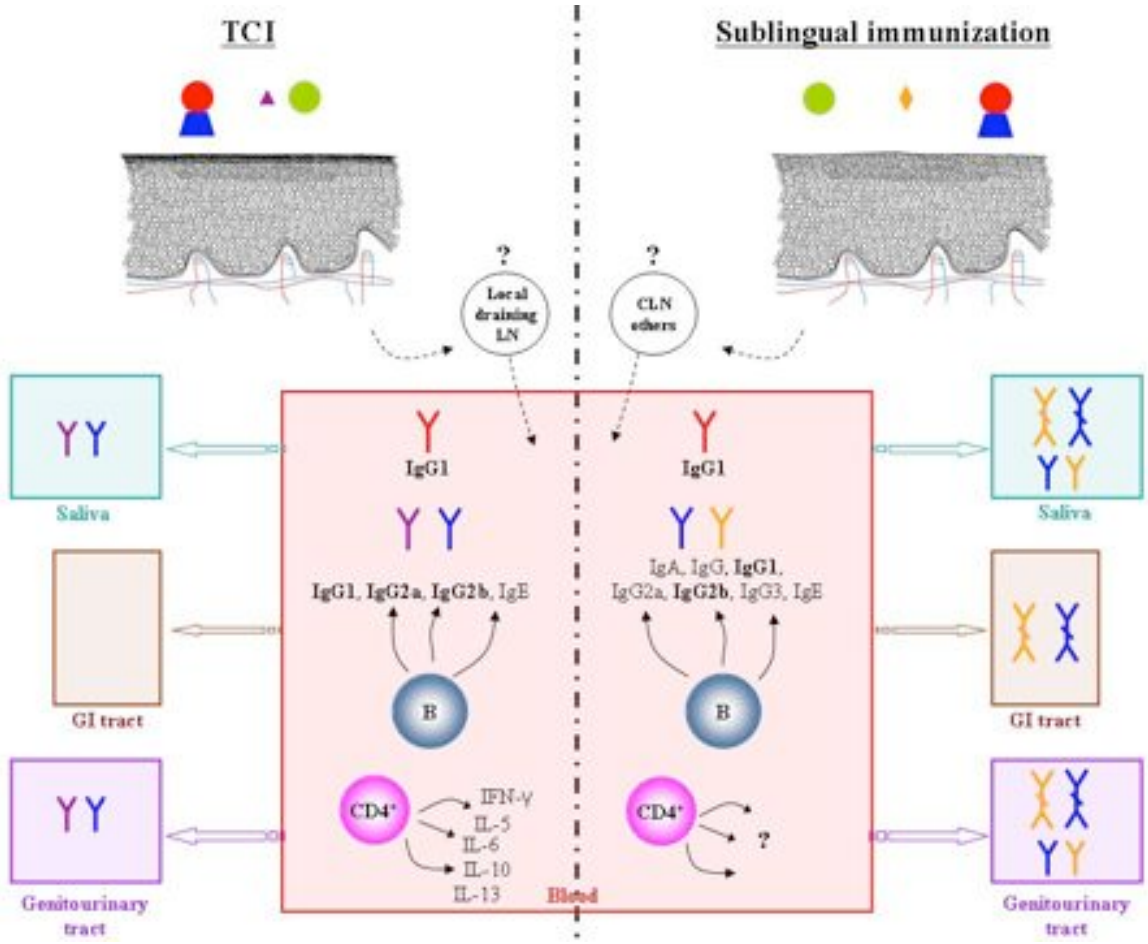

Figure 47. Schematic representations of immune responses after transcutaneous and sublingual immunizations.

Induction of Ag- or adjuvant-specific Ab responses after transcutaneous or sublingual immunizations with Ag plus EdTx are represented. Colors of Abs match the specificity to Ag or adjuvant subunit. Mucosal IgA Abs are represented as dimers with the SC (χ); other mucosal Abs are IgG Abs represented as monomers. CT-specific Abs or Ag-specific responses following CT immunization are not represented. A. Nasal administration of CT leads to accumulation of CT in brain tissues (olfactory bulb and epithelium), after binding to ubiquitous gangliosides receptors, and to inflammation (IL-1, IL-6).

List of publications

8 List of publications

Manuscripts Published

Duverger, A., R. Kobayashi, T. Fukuiwa, K. Fujihashi, D. Tomé, K. Fujihashi, J.R. McGhee, P. N. Boyaka. Non-GM1 ganglioside-targeting bacterial toxins and derivatives with mucosal adjuvant activity. Int J Oral-Med Sci, 2005. 4(2): 53-60.

Duverger, A., R. J. Jackson, F.W. van Ginkel, R. Fischer, A. Tafaro, S. H. Leppla, K. Fujihashi, H. Kiyono, J.R. McGhee, P. N. Boyaka. *Bacillus anthracis* edema toxin acts as an adjuvant for mucosal immune responses to nasally administered vaccine antigens. J Immunol, 2006. 176(3): 1776-83.

Boyaka, P. N., A. Duverger, E. Cormet-Boyaka, and J-N. Tournier. Mucosal Immunity Against Anthrax. Chapter in the Book: Immunity Against Mucosal Pathogens. ed. by Michael Vajdy. 2008. (In press)

Manuscripts in preparation

Duverger, A., S. H. Leppla, W-J. Tang, and P. N. Boyaka. Transcutaneous immunization with anthrax edema toxin derivatives induces IgG Ab responses and cell-mediated immunity. 2008. (In preparation)

Duverger, A., J-M. Carré, W-J. Tang, D. Tomé, S. H. Leppla, and P. N. Boyaka. Contribution of edema factor and protective antigen to the induction of protective immunity by *Bacillus anthracis* edema toxin derivatives as nasal adjuvant. 2008. (In preparation)

Duverger, A., R.J. Jackson, K. Fujihashi, J.R. McGhee, S.H. Leppla, W-J. Tang and P.N. Boyaka. Distinct innate signals are involved in the nasal adjuvant activity of *Bacillus anthracis* edema toxin and cholera toxin. 2008. (In preparation)

Duverger, A., S. H. Leppla, W-J. Tang, and P. N. Boyaka. Mucosal and systemic immune responses mediated by sublingual application of vaccine formulations with edema toxin derivatives. 2008. (In preparation)

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Appendix

Publications

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Invited Review Article

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 stimulation 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 vaccises. These macromolecules are composed of two structurally, functionally, and immunologically separate A and B subunits (1-3). These two AB_5 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 Gsα (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 Gsα (2).

This GTP-binding protein activates adenyl 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 that 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-g-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 dendric 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 repoeted 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, rCT 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 FcepsilonR (common FcRgamma-chain)-deficient and FcgammaRII-deficient mice demonstrated that FcgammaR-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 (38).

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-I 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, GG1b, GD2, GD1a, GM2, GM1	Macrophages (MΦ) Dendritic cells (DC), T cells	Yes	Yes
LT-IIb	GD1a	MΦ, 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	MΦ, DC, T cells	Yes+	Yes

[a]Most 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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Bacillus anthracis Edema Toxin Acts as an Adjuvant for Mucosal Immune Responses to Nasally Administered Vaccine Antigens[1]

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Anthrax edema toxin (EdTx) is an AB-type toxin that binds to anthrax toxin receptors on target cells via the binding subunit, protective Ag (PA). Edema factor, the enzymatic A subunit of EdTx, is an adenylate cyclase. We found that nasal delivery of EdTx enhanced systemic immunity to nasally coadministered OVA and resulted in high OVA-specific plasma IgA and IgG (mainly IgG1 and IgG2b). The edema factor also enhanced immunity to the binding PA subunit itself and promoted high levels of plasma IgG and IgA responses as well as neutralizing PA Abs. Mice given OVA and EdTx also exhibited both PA- and OVA-specific IgA and IgG Ab responses in saliva as well as IgA Ab responses in vaginal washes. EdTx as adjuvant triggered OVA- and PA-specific CD4$^+$ T cells which secreted IFN-γ and selected Th2-type cytokines. The EdTx up-regulated costimulatory molecule expression by APCs but was less effective than cholera toxin for inducing IL-6 responses either by APCs in vitro or in nasal washes in vivo. Finally, nasally administered EdTx did not target CNS tissues and did not induce IL-1 mRNA responses in the nasopharyngeal-associated lymphoepithelial tissue or in the olfactory bulb epithelium. Thus, EdTx derivatives could represent an alternative to the ganglioside-binding enterotoxin adjuvants and provide new tools for inducing protective immunity to PA-based anthrax vaccines. ***The Journal of Immunology,*** **2006, 176: 1776–1783.**

Bacillus anthracis expresses the protective Ag (PA),[4] the lethal factor (LF), and the edema factor (EF), which combine to form two AB-type toxins (1–3). The combination of PA and EF leads to the formation of anthrax edema toxin (EdTx), while lethal toxin (LeTx) results from the combination of PA and LF. The PA subunit targets cells via the anthrax toxin receptor (ATR) 1, which resembles the tumor endothelial marker 8 (4), and the related ATR2, which is similar to the capillary morphogenesis gene 2 (5). The EF is an adenylate cyclase which increases intracellular cAMP levels (6–8) and induces edema (6). Most previous studies have focused on LeTx-induced death and alterations in APC functions in susceptible macrophages (Mϕ) (9–11) and dendritic cells (12). Anthrax toxin-fusion protein derivatives consisting of PA and the N-terminal domain of LF (LF$^{1-254}$) have been used to deliver Ags into the cytosol for presentation via MHC class I molecules and induction of CTL responses (13–16). A recent report suggested that PA may not be needed for intracellular delivery of proteins by the LF N-terminal fragment (17). Intradermal coimmunization 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 Ab responses than immunization with a single plasmid encoding PA (18). Although EdTx was reported to induce accumulation of cAMP in lymphocytes (19) and suppress T cell activation (20), little is known about the effect of EdTx on adaptive immune responses.

Cholera toxin (CT) and the related heat labile toxin I (LT-I) of *Escherichia coli* are AB-type toxins made of pentameric-binding B subunits and enzymatic A subunits with ADP-ribosyl transferase activities (21–23). The B subunits of CT and LT-I bind to GM1 gangliosides on target cells (24), while the more promiscuous B subunit of LT-I also exhibits affinity for GM2 and asialo-GM1 (25–27). CT and LT-I are the best described mucosal adjuvants and both promote mucosal secretory IgA (S-IgA) and plasma Ab responses to coadministered vaccine Ags. Unfortunately, the watery diarrhea induced by these toxins precludes their use as oral adjuvants in humans. In addition, major safety concerns relative to the potential of nasal enterotoxins to target CNS tissues have been reported (28, 29). Thus, nasal enterotoxin could damage CNS tissues in large part through their ADP-ribosyl transferase activity following binding of the B subunit to the promiscuous gangliosides expressed on cells of the CNS (30, 31).

We investigated whether an EdTx derivative could act as a mucosal adjuvant like the enterotoxin CT and LT-I, and promote S-IgA and systemic Ab responses to nasally coadministered vaccine

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[4] Abbreviations used in this paper: PA, protective Ag; LF, lethal factor; EF, edema factor; EdTx, anthrax edema toxin; LeTx, lethal toxin; ATR, anthrax toxin receptor; CT, cholera toxin; LT-I, heat labile toxin-I; S-IgA, secretory IgA; CLN, cervical lymph node; RLU, relative light unit; ON/E, olfactory nerves and epithelium; OB, olfactory bulb; Mϕ, macrophage; NALT, nasopharyngeal-associated lymphoepithelial tissue; CP, crossing point.

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Ags. We also queried whether this regimen would enhance immunity to the binding B subunit PA itself and perhaps provide an extra bonus for anthrax immunity. We further examined whether the receptor specificity of EdTx would lead to the accumulation of this toxin into CNS tissues after nasal delivery.

Materials and Methods

Mice

Female C57BL/6 mice, 6–7 wk of age, were obtained from Charles River Laboratories and were 9–12 wk of age when used in these experiments. All studies were performed in accordance with both National Institutes of Health and University of Alabama at Birmingham institutional guidelines to avoid pain and distress.

Immunization

Mice were nasally immunized three times at weekly intervals with 100 μg of OVA (Sigma-Aldrich) alone, OVA plus 5 μg of rPA only, or OVA plus EdTx (5 μg of rPA together with 5 μg of rEF). The rPA was purified from cultures of a recombinant strain of *B. anthracis* as previously described (32). The EF was obtained from List Biological Laboratories (product no. 173) and was produced in a recombinant strain of *B. anthracis* using an expression plasmid constructed by S. H. Leppla. This EF protein contains a S447N mutation and was shown to display ~20-fold less enzymatic activity than the native EF (33). Controls included mice nasally immunized with OVA plus 1 μg of CT (List Biological Laboratories). Mice were lightly anesthetized and given 12.5 μl of vaccine/nostril. Blood and external secretions (fecal extracts, vaginal washes, and saliva) were collected as previously described (34, 35).

Evaluation of OVA- and PA-specific Ab isotypes and IgG subclass responses

Previously described ELISA was used to assess anti-OVA and anti-PA Ab levels in plasma and external secretions (34, 35). Briefly, microtiter plates were coated with OVA (1 mg/ml) or PA (5 μg/ml). The IgM, IgG, or IgA Abs were detected with HRP-conjugated goat anti-mouse μ-, γ-, or α-H-chain-specific antisera (Southern Biotechnology Associates). Biotin-conjugated rat anti-mouse IgG1 (clone A85-1; 0.5 μg/ml), IgG2a (clone R19-15; 0.5 μg/ml), IgG2b (clone R12-3; 0.5 μg/ml), or IgG3 (clone R40-82; 0.5 μg/ml) mAbs and HRP-conjugated streptavidin (BD Pharmingen) were used to measure IgG subclass responses. The color was developed with the addition of ABTS substrate (Sigma-Aldrich), and the absorbance was measured at 415 nm. End-point titers were expressed as the $\log_2$ dilution giving an OD_{415} of $\geq$0.1 above those obtained with nonimmunized control mouse samples.

Total and Ag-specific IgE Abs

Total IgE Ab levels were determined by a BD OptEIA Set Mouse IgE (BD Pharmingen), according to instructions from the manufacturer. To prevent interference in the assay, serial dilutions of immune plasma were previously depleted of IgG by overnight incubation in Reacti-Bind Protein G-Coated Plates (Pierce). To detect Ag-specific IgE, the microtiter plates were coated with OVA (1 mg/ml) or PA (5 μg/ml). Serial dilutions of plasma were then added, IgE was detected with the biotinylated anti-mouse IgE Abs, and titers were determined as described above.

Macrophage toxicity assay to assess anti-PA-neutralizing Abs

The protective effects of PA-specific Abs were determined using a previously described assay (35) that measures their capacity to protect the J774 Mϕ cell line from LeTx (9, 11). Briefly, J774 Mϕ (5×10^4 Mϕ/well) were added to 96-well, flat-bottom plates. After 12 h of incubation, plasma or external secretions samples were added together with LeTx (400 ng/ml PA plus 40 ng/ml LF) and incubated for an additional 12 h as described elsewhere (31). Viable Mϕ were evaluated after addition of MTT (Sigma-Aldrich) (36).

Effect of EdTx on APCs in vitro

J774 Mϕ (5×10^4 cells/ml) or freshly isolated mesenteric lymph node or spleen cells from C57BL/6 mice were incubated in the presence of PA only (5 μg/ml), EF only (5 μg/ml), EdTx (PA + EF; 5 μg/ml), or CT (1 μg/ml). Forty-eight hours later, culture supernatants were collected for evaluation of cytokine responses. Cells were collected and stained for 30 min at 4°C with FITC- or PE-conjugated mAbs (BD Pharmingen). After three washing steps and fixation in 2% paraformaldehyde, the expression of activation and costimulatory molecules was analyzed by flow cytometry.

In vitro restimulation of Ag-specific CD4$^+$ T cells and cytokine-specific ELISA

T cells were isolated from spleen and cervical lymph nodes (CLNs) and restimulated in vitro as previously described (34, 35, 37) with OVA (1 mg/ml) or PA (20 μg/ml) in RPMI 1640 medium containing 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, 5×10^{-5} M 2-ME, 1 mM sodium pyruvate, and 10% FCS. The Th1 and Th2 cytokines in culture supernatants were determined by cytokine-specific ELISA as previously described (34, 35, 38). The mAb couples were from BD Pharmingen. Standard curves were generated using murine rIFN-γ, rIL-5, rIL-6, and rIL-10, rIL-12 (R&D Systems); rIL-2 and rIL-13 (BD Pharmingen); and rIL-4 (Pierce). The ELISAs were capable of detecting 5 pg/ml for IL-2, IL-4, and IL-5; 15 pg/ml for IFN-γ; 50 pg/ml for IL-13; 100 pg/ml for IL-6, IL-12; and 200 pg/ml for IL-10.

Tracking studies

The PA and CT were labeled with acridinium using an acridinium C_2 NHS ester labeling kit (Assay Designs). The specific activity of PA and CT used in the tracking studies were 2.11×10^7 relative light units (RLU)/ng and 2.06×10^7 RLU/ng, respectively. Mice were given the acridinium-labeled compounds by instilling 5-μl quantities into each nare. Twelve and 24 h following immunization, mice were sacrificed and the olfactory nerves and epithelium (ON/E), olfactory bulbs (OB) and brain (B) were removed as previously described (30). Each tissue was weighed and 200 μl of Cellytic MT lysis buffer (Sigma-Aldrich) was added per 10 mg wet weight of tissue. The ice-cold tissues were homogenized (20,000 rpm for 15–20 s) using a Tissue Tearor (Biospec Products) and frozen at −20°C. After thawing, the homogenates were centrifuged at $10,000 \times g$ for 10 min and the supernatants were tested for light activity in triplicate in 96-well Microlite 2 plates (Thermo Labsystems). Nonimmunized mouse tissues served as controls.

Nasal EdTx-induced cytokine responses in vivo

Mice were given PBS, EdTx or CT by instilling 5-μl quantities into each nare. Twelve and 24 h later, mice were sacrificed and nasal washes were collected in 500 μl of PBS and the cytokine content was analyzed by ELISA. The nasopharyngeal-associated lymphoepithelial tissue (NALT), CLN, ON/E, and OBs were removed and subjected to real time RT-PCR (Lightcycler; Roche). The cytokine mRNA levels are expressed as crossing points (CP) or the cycle at which the fluorescence rises appreciably above the background fluorescence as determined by the Second Derivative Maximum Method (Roche Molecular Biochemicals LightCycler Software). The formula $\text{mRNA} = 2^{(-(\text{CPcytokine} - \text{CP}\beta\text{-actin}))}$ corrects for differences in cDNA concentration between the starting templates of cytokine and housekeeping (i.e., β-actin) genes (39). The simplified formula $20 - (\text{CP}_{\text{cytokine}} - \text{CP}_{\beta\text{- actin}})$ was used to express relative cytokine mRNA responses in tissues of mice given nasal EdTx.

Statistics

The results are expressed as the mean ± 1 SD. Statistical significance (*, $p \leq 0.05$) was determined by Student's *t* test and by ANOVA followed by the Fisher least significant difference test. For statistical analysis, cytokine levels below the detection limit were recorded as one-half the detection limit (e.g., IFN-γ = 7.5 pg/ml).

Results

EdTx promotes plasma Ab responses to nasally coadministered Ags

We first examined whether the adenyl cyclase EdTx, which acts through the ATR, would enhance Ab responses to mucosally administered protein Ags. Coadministration with 1 μg of EdTx (1 μg of PA and 1 μg of EF) enhanced OVA-specific plasma IgG Ab responses (Fig. 1*A*). Higher Ab responses were seen in mice given OVA and 5 μg of PA and 5 μg of EF although the titers failed to reach the statistical difference (Fig. 1*A*). The binding of EdTx to its receptor alone did not significantly contribute to the observed adjuvant activity, because neither PA alone (Fig. 1*B*) nor EF alone (data not shown) significantly increased OVA-specific IgG or promoted OVA-specific IgA Ab responses. Coimmunization with PA and EF (i.e., EdTx) sharply increased OVA-specific IgG and induced high levels of IgA Abs (Fig. 1*B*). The EdTx-induced IgG subclass responses consisted mainly of IgG1 and IgG2b Abs (Fig. 1*C*). Plasma samples collected 1 wk after two nasal immunizations

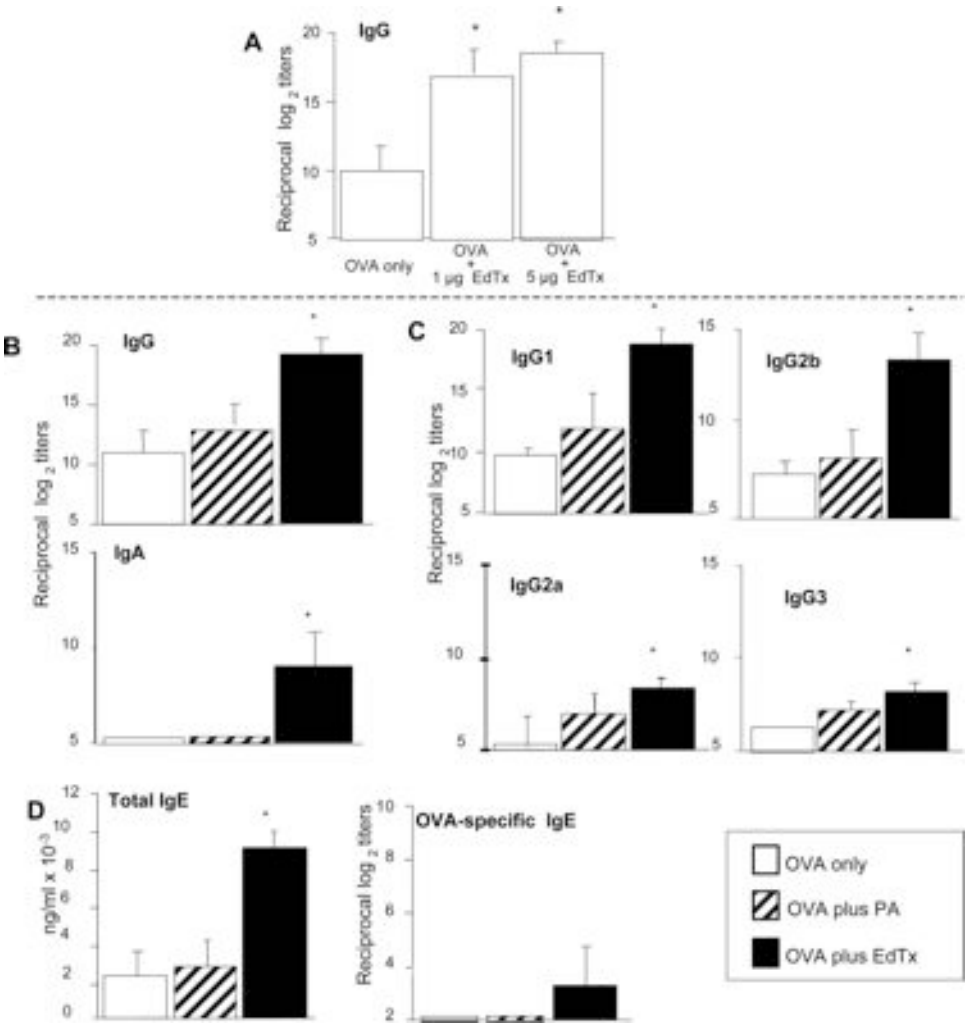

FIGURE 1. Adjuvant effect of EdTx for nasally coadministered Ag. *A*, Effect of EdTx dose on OVA-specific Ab responses. Mice were immunized three times at weekly intervals with 100 μg of OVA only or OVA plus 1 or 5 μg of EdTx (5 μg of PA plus 5 μg of EF). *B*, Role of EdTx component in the adjuvanticity. *C*, Plasma levels of OVA-specific IgG subclass Abs were determined 1 wk after the last immunization. *D*, Plasma samples were collected 1 wk after the second immunization (day 14) and examined for their content of total and OVA-specific IgE Ab levels. Mice were immunized three times at weekly intervals with 100 μg of OVA only (□), OVA plus 5 μg of PA (▨), or OVA plus 5 μg of EdTx (5 μg of PA plus 5 μg of EF) (■). Plasma levels of OVA-specific Abs were determined 1 wk after the last immunization. The results are expressed as the reciprocal $\log_2$ titers ± one SD and are from three (*A*) or five experiments (*B–D*) and four mice/group. *, $p < 0.05$.

with OVA and EdTx contained elevated levels of total IgE Abs and low but significant OVA-specific IgE Abs that were not detected in mice nasally immunized with OVA only or OVA plus PA (Fig. 1*D*).

EdTx enhances PA-specific plasma Ab responses

In addition to acting as adjuvants for mucosally coadministered Ags, CT and LT-I are good immunogens that induce high Ab responses to their respective cell ganglioside-binding B subunits (40–43). Thus, we next addressed whether EdTx could enhance immunity to its binding PA subunit. Low levels of PA-specific IgG and IgA Abs were seen in the plasma of mice that received 5 μg of PA. Interestingly, PA-specific IgG and IgA Ab responses were both significantly increased in mice given EdTx (5 μg of PA plus 5 μg of EF), indicating that the enzymatic EF subunit also enhanced PA-specific Ab responses (Fig. 2*A*). PA-specific IgE Abs were not detected in mice that received PA without EF. However, coadministration of EF induced PA-specific IgE Ab levels (Fig. 2*A*), which were significantly higher than OVA-specific Abs in the same mice (Fig. 1*C*). The EdTx-induced PA-specific IgG subclass responses also predominantly consisted of IgG1 and IgG2b Abs followed by IgG2a and IgG3 (Fig. 2*B*).

EdTx induces PA- and OVA-specific Abs in saliva and vaginal secretions

Because nasal administration of EdTx enhanced systemic Ab responses to both the coadministered protein (i.e., OVA) and the binding B subunit (i.e., PA) itself, we next ascertained whether EdTx also induced PA- and OVA-specific IgA and IgG Abs in saliva and mucosal IgA Ab responses in other external secretions. OVA-specific IgA Abs were not detected in the saliva or the vaginal secretions of mice given OVA only or OVA plus PA (Fig. 3*A*). In contrast, the saliva of mice that received EdTx exhibited both OVA- and PA-specific IgA and IgG Abs (Fig. 3*A*). In addition, PA- and OVA-specific IgA Abs were detected in the vaginal washes of mice immunized with OVA plus EdTx (Fig. 3*B*).

Previous studies have shown that CT as a nasal adjuvant could induce a broad mucosal Ab response with PA-specific IgA Abs in the oral cavity (saliva), gastrointestinal (fecal extracts), and genitourinary (vaginal washes) tracts (35). In contrast to CT, the adjuvant activity of EdTx failed to induce high OVA-specific IgA Abs in fecal extracts (Fig. 3*C*). High amounts of PA (i.e., 40 μg) were needed for CT as a nasal adjuvant to induce anti-PA IgA Abs in fecal extracts (35). Increasing the dose of PA to 40 μg failed to enhance PA- or OVA-specific IgA Abs in fecal extracts (data not shown), suggesting that different mechanisms are involved in the mucosal adjuvanticity of CT when compared with EdTx.

EdTx induces anti-PA-neutralizing Abs

Nasal immunization with OVA and PA resulted in low levels of neutralizing Abs that were seen only in the plasma (Table I). Mice immunized with OVA and EdTx exhibited significantly higher titers of neutralizing Abs in the plasma than mice immunized with OVA plus PA (Table I). More interestingly, saliva of mice given

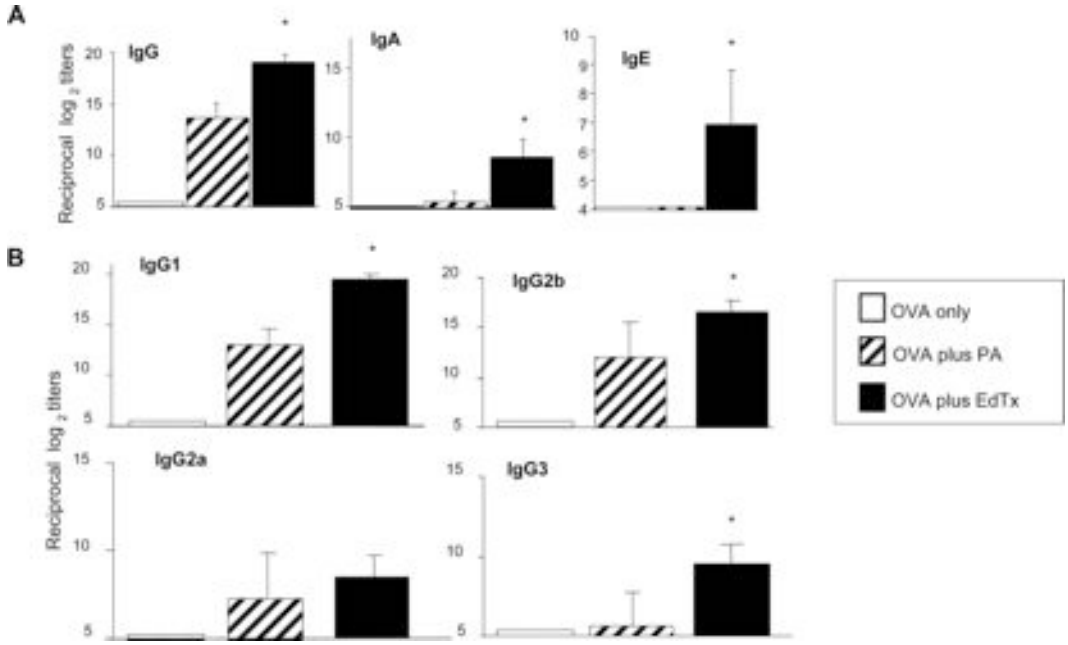

FIGURE 2. Plasma PA-specific Ab responses following nasal immunization with EdTx as adjuvant. Mice were immunized three times at weekly intervals with 100 μg of OVA only (□), OVA plus 5 μg of PA (▨), or OVA plus 5 μg of EdTx (5 μg of PA plus 5 μg of EF) (■). Plasma PA-specific IgE Ab responses were determined in samples collected at day 14, and plasma PA-specific Ab isotype (*A*) and IgG subclass responses (*B*) were determined 1 wk after the last immunization. The results are expressed as the reciprocal $\log_2$ titer ± one SD from five separate experiments and four mice per group per experiment. *, $p < 0.05$

OVA and EdTx contained significant levels of anti-PA neutralizing Abs (Table I). No significant neutralizing Abs were detected in vaginal washes (Table I).

Ag-specific T cell cytokine responses following nasal immunization with EdTx as adjuvant

To characterize $CD4^+$ Th cell cytokine pathways associated with EdTx-induced immunity, we examined the pattern of cytokines secreted by OVA- and PA-specific $CD4^+$ T cell after a 5-day in vitro restimulation. Spleen $CD4^+$ T cells from mice immunized with OVA and EdTx secreted mixed Th1- and Th2-type cytokines after in vitro restimulation with OVA or PA as indicated by high levels of IFN-γ (Th1) but also IL-5, IL-6, and IL-13 in culture supernatants (Table II). IL-4 levels were below the limit of detection. The same profile of cytokine responses was seen in the culture supernatant of CLN $CD4^+$ T cells restimulated under the same conditions (data not shown).

Effect of EdTx on cytokine secretion and costimulatory molecule expression by Mϕ, in vitro

We assessed the direct effects of EdTx or CT on the expression of costimulatory molecules (i.e., CD40 and CD86) as well as cytokine secretion by J774 Mϕ to address whether EdTx regulates APC functions. Consistent with previous reports (44–46), CT induced high levels of IL-6 responses in treated cells (Fig. 4*A*) and increased costimulatory molecule expression (Fig. 4*B*) by Mϕ in vitro. The IL-6 responses after EdTx stimulation were ~10-fold lower than those measured in culture supernatants of J774 Mϕ cultured in the presence of CT (Fig. 4). However, EdTx up-regulated the expression of both CD40 and CD86 by J774 Mϕ to the same extent as the mucosal adjuvant CT. These findings were further confirmed on T cell-depleted spleen cells (data not shown).

Nasal PA does not target CNS tissues

We next explored the possibility that EdTx could target the ON/E or other CNS tissues and induce inflammatory responses. Fig. 5*A*

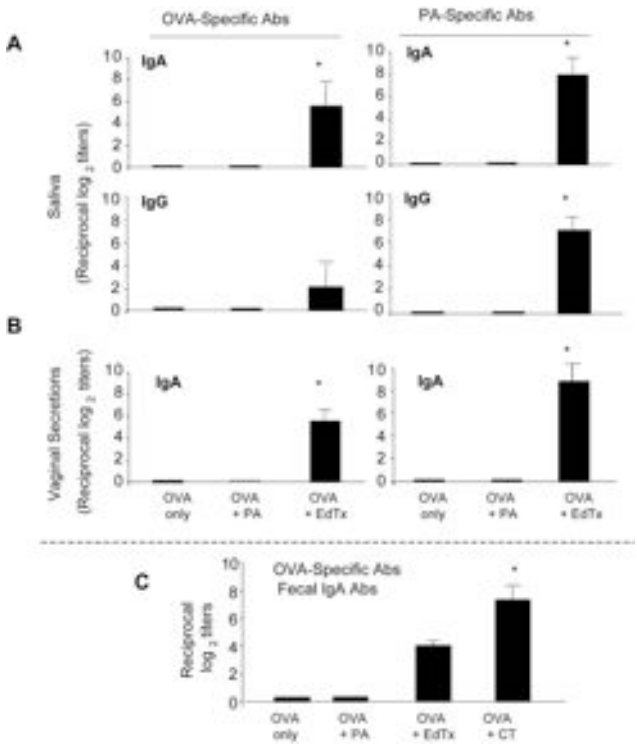

FIGURE 3. Mucosal IgA Ab responses following nasal immunization with EdTx as adjuvant. Mice were immunized three times at weekly intervals with 100 μg of OVA only, OVA plus 5 μg of PA, or OVA plus 5 μg of EdTx (5 μg of PA plus 5 μg of EF). Saliva (*A*) and vaginal washes (*B*) were collected 2 wk after the last immunization. The Ab levels are expressed as the reciprocal $\log_2$ titer ± 1 SD from five separate experiments and four mice per group per experiment. Fecal extracts (*C*) were collected 2 wk after the last immunization with either OVA only, OVA plus PA, OVA plus EdTx, or OVA plus CT. The IgA Ab levels were expressed as the reciprocal $\log_2$ titer ± 1 SD from three separate experiments and four mice per group per experiment. *, $p < 0.05$

Table I. *Neutralizing anti-PA Abs produced in response to nasal administration of EdTx protect Mϕ against the lethal effect of LeTx in vitro*[a]

Immunization	Neutralizing Ab Titers (1/Dilution)		
	Plasma	Saliva	Vaginal Washes
OVA only	BD	BD	BD
OVA plus PA	8,192 ± 256*	BD	BD
OVA plus EdTx	131,072 ± 1,024*	10 ± 3*	3 ± 2

[a] Serial dilutions of each sample were added to J774 Mϕ cultures in the presence of LeTx. The neutralizing titers were determined as the last dilution yielding an MTT OD equal to twice the background value. Results are shown as neutralizing Ab titers and are expressed as the reciprocal dilutions titers ± 1 SE of three separate experiments and five mice per group per experiment. BD, Below detection level; *, $p < 0.05$.

illustrates the failure of PA to accumulate in the ON/E 24 h following nasal delivery of 5 μg of PA. In addition, there was no PA detectable in the OBs or brain and only minor amounts (< 0.15 ng/10 mg tissue) were observed in the NALT, CLN, or spleen 24 h after nasal delivery (data not shown). Nasal PA given together with EF (EdTx) did not increase PA accumulation in olfactory or brain tissues (Fig. 5*A*). In contrast to PA or EdTx, nasal delivery of a 0.5-μg dose of CT resulted in significant accumulation in the ON/E which was further increased when the CT dose was 10-fold higher (Fig. 5*A*). We also found that nasal CT, but not EdTx, up-regulated IL-1 mRNA levels in the ON/E and in the NALT suggesting that CT targeted these two sites (Fig. 5*B*). Finally, mice given nasal CT, but not those given nasal EdTx, exhibited high levels of IL-6 in nasal washes (Fig. 5*C*).

Discussion

The enterotoxins CT and LT-1, which deliver their ADP ribosyl transferase A subunit via ganglioside targeting, are well-recognized adjuvants for induction of mucosal immunity to coadministered Ags (40–42). The CTA1-DD molecule which targets CT-A to B cells was also shown to be an effective mucosal adjuvant (31, 47, 48). EdTx 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. In this study, we show that EdTx promotes both systemic and mucosal adaptive immunity to nasally coadministered Ags and enhances PA-specific Ab responses significantly above levels achieved by administration of PA without EF. We also show that ATR targeting by EdTx did not lead to the accumulation of this adjuvant into olfactory and CNS tissues.

Anthrax toxin derivatives have been evaluated as molecular syringes for intracellular delivery of peptides (13, 51) or protein Ags (14–16) for presentation via the MHC class I pathway and induction of cytotoxic $CD8^+$ T cells. Our results show that in vivo delivery of anthrax EdTx provides necessary signals for induction of mucosal and systemic immunity to coadministered protein Ags. Although data summarized in this manuscript only referred to nasal delivery of EdTx, we also found that EdTx is an adjuvant for protein Ags coinjected i.p. in both C57BL/6 and BALB/c mice (data not shown). Studies with the ganglioside-targeting enterotoxins CT and LT-I (52–55) and derivatives including their chimeras (34, 56) or the B cell-targeting CTA1-DD (31, 57) have demonstrated the importance of receptor binding for controlling the immune responses induced by these ADP-ribosylating adjuvants. Thus, the adjuvant activity of CT appears to be more dependent on IL-4 and $CD4^+$ Th2 cell cytokines (52, 53). In contrast, the more promiscuous LT-I, which binds GM1 gangliosides like CT but also asialo-GM1 and -GM2 gangliosides, promotes a broader spectrum of responses with $CD4^+$ Th cells producing both IFN-γ and Th2-type cytokines (34, 54). The adjuvant activity of nasal EdTx appears to involve Ab and T cells responses that resemble those induced by LT-I rather than CT. Thus, EdTx induced $CD4^+$ T cells secreting both IFN-γ and Th2-type cytokines. Further, EdTx as an adjuvant promoted only modest levels of OVA-specific IgE ($\log_2$ titers = 3) when compared with those seen after nasal immunization with CT (i.e., $\log_2$ titers = 8). Although it has been suggested that ATRs may not be expressed at the apical membrane of epithelial cells (50), there is no information to date on the relative expression of ATRs on immune cells and potential cellular targets of nasally administered EdTx. We have shown here that PA alone or as a component of EdTx does not target olfactory tissues and do not induce IL-1-specific mRNA in ON/E. It is also important to note that EdTx promotes a similar profile of serum Ab responses than CTA1-DD which targets B cells (31).

The mechanisms underlying the induction of mucosal immunity and S-IgA Ab responses by bacterial toxins remain only partially understood. Studies over the past two decades have shown a role for CT-induced cytokines on its mucosal adjuvanticity. Thus, CT was shown to induce both IL-6 and IL-1 secretion by epithelial cells and APCs (44, 58–60). Both IL-1 and IL-6 were later shown to be adjuvants for systemic immunity to nasally coadministered protein vaccines (38, 61) and IL-1 was also able to promote mucosal IgA Ab responses (61). Other factors thought to contribute to the adjuvanticity of CT include its ability to up-regulate the expression of MHC (44) and costimulatory molecules (45, 46, 62).

Table II. *Ag-specific $CD4^+$ T cell cytokine responses induced by nasal EdTx as an adjuvant*[a]

Immunization	In vitro Stimulation	Cytokines (pg/ml)			
		IFN-γ	IL-5	IL-6	IL-13
OVA only	None	156 ± 30	BD	36 ± 1	20 ± 2
	OVA	1320 ± 49*	41 ± 1*	175 ± 10*	36 ± 6
	PA	189 ± 10	BD	31 ± 1	20 ± 0
OVA plus EdTx	None	267 ± 37	BD	31 ± 1	20 ± 0
	OVA	9666 ± 1524*	418 ± 85*	386 ± 16*	188 ± 29*
	PA	16550 ± 2557*	1340 ± 160*	192 ± 28*	406 ± 78*

[a] Spleen $CD4^+$ T cells were isolated 21 days after the initial immunization and restimulated in vitro with OVA (1 mg/ml) or PA (20 μg/ml). Culture supernatants were collected after 5 days and cytokines evaluated by ELISA. The results are expressed as the mean ± 1 SE and are representative of four separate experiments. The same profile of responses was seen with $CD4^+$ T cells isolated from corresponding cervical lymph nodes. BD, Below detection levels; *, $p < 0.05$

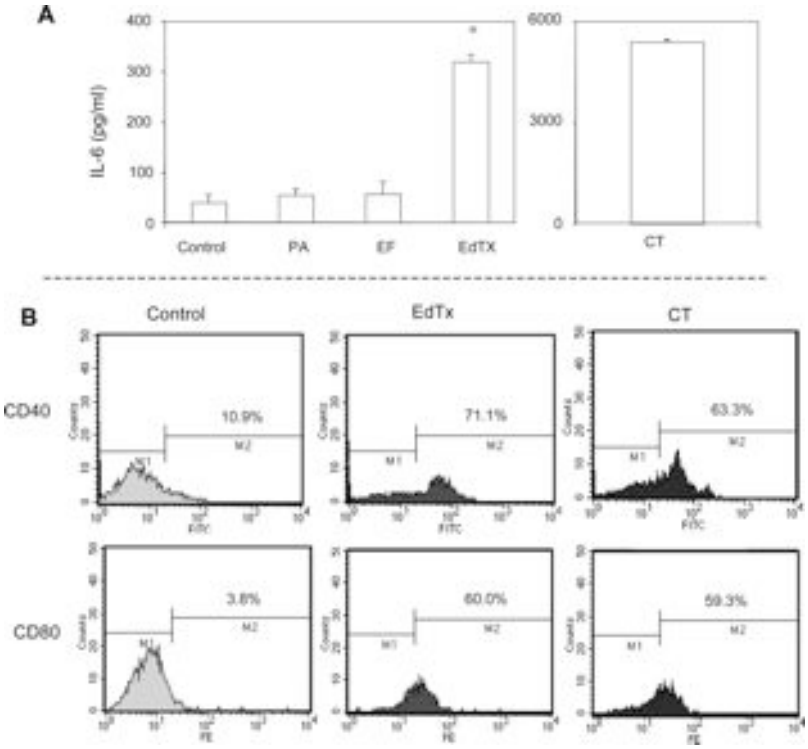

FIGURE 4. Effects of EdTx on cytokine secretion and costimulatory molecule expression by Mϕ. The J774 Mϕ were cultured for 48 h in the presence of PA (5 μg/ml), EF (5 μg/ml), EdTx (5 μg/ml PA + 5 μg/ml EF), or CT (1 μg/ml). *A*, Cytokine secretion was analyzed in culture supernatants, and results were expressed as mean $\pm$ 1 SD of four separate experiments (*, $p < 0.05$). *B*, Cells were analyzed by flow cytometry for expression CD40 and CD86. Results are representative of four separate experiments.

We have shown that EdTx stimulates IL-6 secretion by Mϕ cultures and enhances the expression of costimulatory molecules. We should stress that IL-6 levels induced by EdTx were ~10-fold lower than those seen in Mϕ cultures stimulated with the same dose of CT. The lower stimulatory effect of EdTx for IL-6 secretion was further confirmed in vivo where nasal delivery of CT but not EdTx induced IL-6 secretion in nasal washes. It is unlikely that the reduced ability of EdTx to induce IL-6 (and IL-1) could alone explain the polarized Ag-specific mucosal IgA Abs responses when compared with those generally induced by CT (34, 46, 63) or LT as adjuvants (34, 46, 54, 56). The role of the adenylate cyclase activity for the adjuvanticity remains to be elucidated. In this regard, the EF used in our studies contains a S447N mutation that could account for its recently reported 20-fold lower ability to

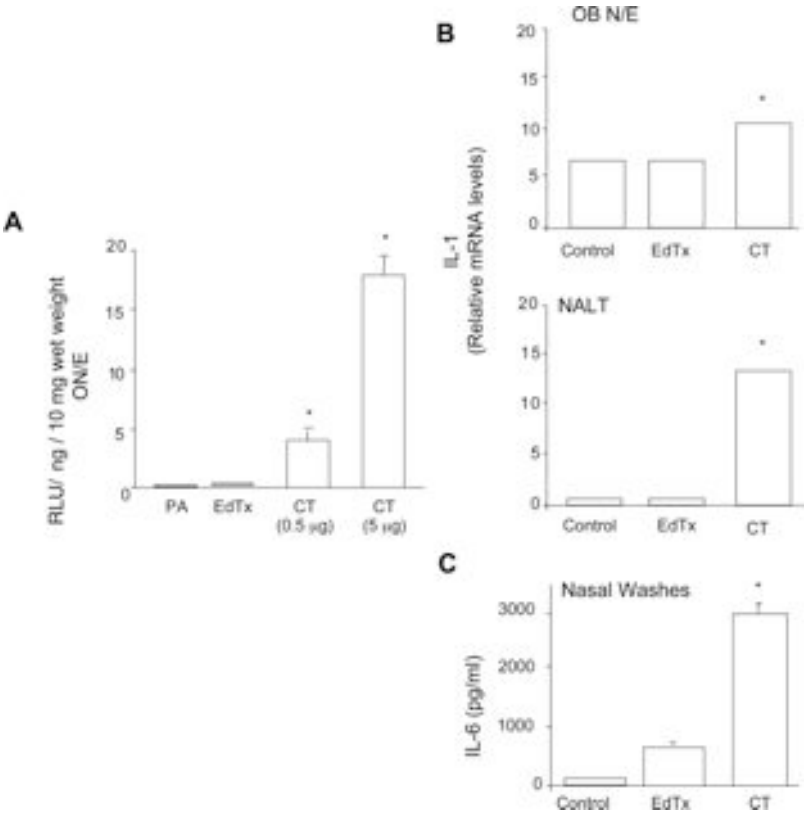

FIGURE 5. *A*, Tracking uptake into olfactory and CNS tissues. Groups of mice were given acridinium-labeled PA (5 μg), EdTx (5 μg of PA plus 5 μg of EF), or CT (0.5 or 5 μg) by the nasal route. *A*, Twenty-four hours later, mice were sacrificed and the ON/E and OBs were collected, homogenized, and tested for light activity as described in *Materials and Methods*. Results are expressed as mean RLU per nanogram of individual tissues $\pm$ 1 SD and are from two separate experiments with three mice per group (*, $p < 0.05$). *B*, IL-1 mRNA analysis by real-time RT-PCR. Results are expressed as mean relative mRNA levels from triplicate assays performed with pooled tissues (three mice per group) and are representative of three separate experiments (*, $p < 0.05$). *C*, Analysis of IL- 6 levels in nasal washes by ELISA. Results are expressed as picograms per milliliter $\pm$ 1 SD of IL-6 levels in individual nasal washes and are from two experiments with three mice per group. *, $p < 0.05$ when compared with controls given PBS.

induce cAMP when compared with recombinant native EF protein (33). Several mutants of both CT and LT-I, which are devoid of ADP-ribosyl transferase activity, were shown to retain their mucosal adjuvant activity and the ability to promote high mucosal IgA Ab responses. In another system, the mucosal adjuvant activity of CTA1-DD was reported to require both effective B cell targeting and the ADP ribosyl transferase activity (57). Thus, further studies are warranted to establish the mechanisms of mucosal adjuvanticity of EdTx.

Enterotoxin adjuvants are also potent immunogens which induce elevated immune responses to their binding subunits (41, 43). A major finding of this study resides in the fact that EdTx is a potent immunogen and that the presence of EF increased anti-PA responses above levels achieved after administration of PA alone. We have previously reported that high levels of PA-specific Abs could be detected in mouse plasma following nasal immunization with PA doses of up to 25 μg when CT was used as adjuvant (35). The same studies showed that 40 μg of PA/dose were needed to promote PA-specific mucosal IgA Abs. The results summarized here indicated that ATR targeting with much lower doses of PA (i.e., 5 μg) and EF allows the induction of PA-specific S-IgA Abs. Interestingly, increasing the dose of PA given with EF to 40 μg did not increase the levels of PA-specific mucosal IgA Abs, suggesting that codelivery of EF achieved optimal S-IgA Ab responses with low nasal dose of PA. Although, nasal immunization with CT or LT-I as adjuvant primarily promotes IgA Ab responses in the respiratory and genitourinary tracts, low but significant responses are consistently seen in the gastrointestinal tract. PA was previously reported to deliver a functionally active CT-A subunit into mammalian cells using an LF^{1-254}-CT-A fusion protein (64). Therefore, it will be interesting to examine whether the ATR-mediated cellular targeting, the adenylate cyclase, and possibly other activities of EF govern the polarized PA-specific mucosal IgA Ab responses induced by EdTx as adjuvant.

In summary, we have shown that ATR targeting of an adenylate cyclase subunit provides an effective strategy for enhancing the immune response to nasal vaccines. The EdTx as nasal adjuvant does not target the olfactory or other CNS tissues and thus could represent a safer alternative to ganglioside-binding adjuvants. In addition, EdTx very efficiently promoted anti-PA Ab responses both in saliva and in plasma and this could have important implications for improving the efficacy of current PA-based anthrax vaccines.

Disclosures

The authors have no financial conflict of interest.

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Chapter 13
Mucosal Immunity Against Anthrax

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Abstract Anthrax has recently gained much attention due to the potential use of *Bacillus anthracis* spores as an agent of biowarfare. In the event of intentional dissemination of *Bacillus anthracis* spores, the most likely routes of infection are through the nasopharyngeal and gastrointestinal tracts. Since inhalational and gastrointestinal anthrax are the most severe and potentially lethal forms of the disease, appropriate vaccine strategies are needed to provide optimal protection against these mucosally initiated infections. This chapter will review the role of anthrax toxins in the pathogenesis of anthrax, with special emphasis on how *Bacillus anthracis* lethal toxin and edema toxin affect immune cells and alter innate and adaptive immunity. We also will summarize traditional strategies for vaccination against anthrax and discuss new approaches to inducing both mucosal and systemic immunity against anthrax toxins for enhanced protection against inhalational and gastrointestinal anthrax.

13.1 Introduction to *Bacillus Anthracis* and the Disease Anthrax

The Gram-positive, spore forming, rod shaped *Bacillus anthracis* produces a poly-γ-D-glutamic acid capsule attached to the peptidoglycan of the cell wall and a tripartite exotoxin for initiation of the clinical manifestations of anthrax [1, 2, 3]. The capsule enables the recently germinated anthrax *Bacillus* to resist phagocytic destruction [2]. It also provides a poorly immunogenic surface vital for full virulence. The three-components exotoxin produced is thought to contribute directly to the lethality of anthrax [1, 2, 3]. *Bacillus anthracis* infection leads to three forms of diseases depending on the cutaneous, gastrointestinal or inhalational route of exposure to *Bacillus anthracis* spores. Cutaneous anthrax traditionally has been reported in agricultural settings and this form of infection rarely evolves into a

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lethal disease. The gastrointestinal and inhalational forms of diseases traditionally have occurred among individuals in close contact with animal or animal products. These two mucosal routes of infection generally are associated with more severe symptoms than cutaneous infectious and often lead to death. Thus, biowarfare and bioterrorist development of anthrax focuses on inhalation and ingestion of anthrax spores.

13.2 Gastrointestinal Anthrax

Cases of gastrointestinal anthrax occur in areas endemic for anthrax, which exist in tropical and subtemperate regions. Contamination often results from consumption of undercooked meat or meat product. It also is believed that cases of gastrointestinal anthrax may have resulted from the consumption of anthrax-containing chocolate developed as biological weapons during World War II [4]. Despite the wide geographic distribution of anthrax endemicity, detailed epidemiologic data on gastrointestinal anthrax are rare. This mostly is due to lack of microbiologic, pathologic, or serologic testing in developing countries. Based on limited reports of gastrointestinal anthrax, it appears that the he disease develops a few days after ingesting spore-contaminated meat. The spectrum of the resulting disease ranges from asymptomatic to nausea, vomiting, abdominal pain, bloody diarrhea and even death by shock or sepsis. Ingested anthrax spores can cause lesions in all segments of the gastrointestinal tract from the oral cavity to the coecum. Ulcerative lesions, usually multiple and superficial, may occur in the stomach, and possibly, in association with similar lesions of the esophagus and jejunum [5, 6]. These ulcerative lesions may bleed, resulting in massive and fatal hemorrhage in severe cases [5, 6]. Lesions also were reported farther down the gastrointestinal tract, in the mid-jejunum, terminal ileum, or cecum. These lesions of the lower gastrointestinal tract tend to develop around a single site or a few sites of ulceration and edema. In addition, these lesions may lead to hemorrhage, obstruction, perforation, or any combination of these symptoms [7]. Pathologic examination of gastrointestinal anthrax showed lesions on the mucosa, as well as in mesenteric lymph nodes which are enlarged and become hemorrhagic [7]. A bioluminescent *Bacillus anthracis* was recently constructed to examine the site of bacteria multiplication after ingestion of spores. These bacteria provide a unique experimental system where strongly luminescent bacteria can easily be discriminated from nonluminescent dormant spores. This system has helped demonstrate the presence of bacterial rods, numerous polymorphonuclear cells, necrosis, and hemorrhage in the Peyer's patches of mice orally administered *Bacillus anthracis* spores [8]. Thus, following ingestion of *Bacillus anthracis* spores, bacterial growth occurs primarily in Peyer's patches [8]. This study showed that spores are continuously shed for up to 48 h post-inoculation after a majority of the initial inoculum has been excreted. It also demonstrated that anthrax spores germinate in the gastrointestinal tract and that vegetative *Bacillus anthracis* transiently colonize the intestinal lumen [8].

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13.3 Inhalational Anthrax

Inhalational anthrax was known in the 1800s and early 1900s as a disease related to the textile (woolsorters) and tanning industries. The largest outbreak of inhalational anthrax documented in the 20th century occurred in Sverdlovsk in the former Soviet Union, as a result of aerosol release of *Bacillus anthracis* spores from a military facility [9, 10]. Less than two dozen cases of inhalational anthrax were reported in the US before the intentional release of *Bacillus anthracis* spores in 2001 [11]. In contrast to the gastrointestinal form of disease, more epidemiological studies are available on inhalational anthrax. It is well established that inhalation anthrax is a biphasic illness characterized by a silent initial phase of 1 to 4 days followed by a fulminant phase. The initial silent phase is associated with flu-like symptoms including malaise, fatigue, fever and non-productive cough. Symptoms of the fulminant phase, which can be followed by death, include respiratory distress, bluish discoloration of the skin or mucous membranes caused by lack of oxygen in the blood, and excessive sweating. It is important to note that nausea and vomiting also were observed in the initial phase of disease among the recent case of inhalational anthrax in the US [11]. This finding could indicate an early involvement of the gastrointestinal tract after aerosol infection.

The current model of inhalational anthrax considers that anthrax spores are take-up by alveolar macrophages and transported to the draining mediastinal lymph nodes, where they germinate and establish infection within the lymphatics before the vegetative forms ultimately disseminate via the general circulation. Recent studies have confirmed the role of alveolar macrophages in the uptake of *Bacillus anthracis* spores. However, it now appears that dendritic cells also can endocytose *Bacillus anthracis* spores [12], and interstitial lung dendritic cells were identified as the cell population that transports spores into the thoracic lymph nodes [13]. Since spores were seen in lung dendritic cells up to 72 h after intranasal infection [13], the current view that inhaled spores of *Bacillus anthracis* do not germinate before they reach the draining lymph nodes is in question. In this regard, studies with recombinant bioluminescent *Bacillus anthracis* have shown that inhaled spores establish initial infection in nasal-associated lymphoid tissues [8]. Thus far, inhalational anthrax has been well-accepted as leading to an infection of the general bloodstream. It now appears that this form of infection includes a significant mucosal component, against which mucosal immunity likely is required for optimal protection of the host.

13.4 *Bacillus Anthracis* Exotoxins

Bacillus anthracis produces a tripartite exotoxin, which consists of the protective antigen (PA), the edema factor (EF) and the lethal factor (LF) [14]. The PA subunit targets cells via the anthrax toxin receptor 1 (ATR1), which resembles the tumor endothelial marker 8 (TEM8) [15], and the related ATR2, which is similar to the capillary morphogenesis gene 2 (CMG2) [16]. After the 83 kDa PA (PA83) binds to the ATR1/TEM8 or ATR2/CMG2, a protease furin cleaves a 20 kDa peptide (PA20) [17]

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which allows the formation of a PA63 heptamer in the host cell membrane [18]. This PA heptamer ring serves to anchor EF, LF or the two in combination [19]. The three exotoxins are not toxic separately, but only in combination. Thus, the combination of PA and EF is usually termed edema toxin (EdTx), while the combination of PA and LF is termed lethal toxin (LeTx). These names originate from the early observations that intravenous injection of PA plus LF is lethal [20] and that intradermal injection of PA plus EF induces edema in the skin [21]. It has been suggested that the low-density lipoprotein receptor-related protein 6 (LRP6) is a co-receptor with either ATR1/TEM8 or ATR2/CMG2, which promotes endocytosis of anthrax toxin into cells [22]. However, this role of LRP6 has been challenged recently [23]. The LF has been crystallized [24] and was shown to be a protease, which cleaves mitogen-activated protein kinase kinase (MAPKK) and thus inhibits intracellular signaling [25]. On the other hand, EF is an adenylate cyclase [26] whose structure has been solved [27]. After binding to calmodulin, EF undergoes a molecular rearrangement to acquire its adenylate cyclase activity and elevate intracellular cyclic adenosine monophosphate (cAMP) levels [27].

13.4.1 Anthrax Lethal Toxin Alters Innate and Adaptive Immune Functions

A number of studies have established that LeTx contributes to the pathogenesis of anthrax by altering both the innate and adaptive branches of host immunity. In fact, LeTx is known to inhibit the secretion of proinflammatory cytokines by macrophages [28, 29, 30]. In more recent studies, LeTx was shown also to inhibit the secretion of all cytokines by dendritic cells [31]. However, others observed more selective effects of LeTx on cytokine secretion by dendritic cells, which correlated with a selective inhibition of p38 and ERK signaling [32]. More specifically, this study has shown that LeTx inhibits DCs production of IL-10 and TNF-α, but not IL-12p70 [32]. Phospholipase A2 type IIA expression was demonstrated to be crucial for killing of anthrax spores by pulmonary phagocytes [33]. LeTx also alters innate immunity by inhibiting the production of phospholipase A2 type IIA thus, inhibiting the bactericidal activity of phagocytes [33]. A recent study has shown that LeTx could inhibit the bactericidal activity of alveolar macrophages in non-human primate [30]. In addition, LeTx has been shown to prevent chemotaxis of polymorphonuclear cells. One potential mechanism for alteration of chemotaxis includes MKK-1-independent signaling and the inhibition of actin polymerization [34]. The two other mechanisms involve reduction or switch-off of chemokine production by dendritic cells [12], and inhibition of chemokine receptor expression on responding cells with subsequent blocking of chemotaxis [35].

In addition to affecting innate immune responses, LeTx can alter the development of adaptive immune responses. For example, LeTx was reported to impair the ability of antigen presentation by LPS-stimulated dendritic cells through inhibition of MHC and co-stimulatory molecule expression [31, 36]. Furthermore, LeTx has been shown to inhibit antigen-specific antibody responses [31]. It has become clear

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that the inhibitory effects of LeTx on adaptive immune responses occur via direct effects on both T- and B-lymphocytes. LeTx inhibits mouse lymphocyte activation in vivo [37] and blocks IL-2 production by $CD4^{+}$T cells [38]. LeTx also has been reported to block MKK-dependent B-cell proliferation and IgM production [39].

The best described role of LeTx is certainly its ability to induce the death of macrophages [40, 41, 42, 43, 44, 45] and dendritic cells [46]. Interestingly, several studies have shown that MKK cleavage occurs in both resistant and susceptible mouse cells [42, 46], suggesting that LeTx-induced cell death is independent of MKK cleavage. In this regard, one study suggested that LeTx induces apoptosis of activated macrophages via a p38a-dependent pathway [42, 47]. Another apoptosis pathway, involving the activation of PKR downstream from TLR4 by *Bacillus anthracis* motifs recently has been described [48]. Of interest, the susceptibility of mouse macrophages to LeTx recently has been linked to a polymorphism in the locus *Nalp1b*, which encodes NALP1 [49]. In fact, NALP1 activation only occurs in macrophages susceptible to LeTx [49]. The NALP1 is a key component of the inflammasome or the multiprotein complex responsible for the activation of caspases 1 and 5, leading to the processing and secretion of the pro-inflammatory cytokines IL-1**β** and IL-18 or/leading to a potent inflammatory response. Therefore, LeTx-induced death could involve the activation of caspases, alone or together with secretion of pro-inflammatory cytokines [50].

13.4.2 Effect of Anthrax Edema Toxin on Innate and Adaptive Immune Functions

Until recently, little was known about the role played by EdTx in the alteration of host immune functions during anthrax infection. EF, the enzymatic moiety of EdTx, binds/utilizes ATP and alters the functions of macrophages. In fact, it now is evident that EdTx alone or in combination with LeTx affects innate immunity. Thus, EdTx inhibits LPS-induced oxidative burst [51] and other functions of neutrophils [34, 52]. EdTx activates the expression of activator protein-1 (AP-1) and CAAAT/enhancer-binding protein-beta (C/EBP-beta) by macrophages, but also inhibits their phagocytic ability and production of TNF-α [53]. It has been shown that EdTx selectively inhibits the production of IL-12p70 by murine dendritic cells in vitro, but also, secretion of IL-12p70 and IFN-γ secretion induced by LPS, in vivo [32]. Both LeTx and EdTx inhibit TNF-α production by dendritic cells, but in contrast with LeTx, EdTx does not inhibit production of the anti-inflammatory cytokine IL-10 [32]. In summary, while anthrax spores stimulate proinflammatory cytokine responses by macrophages and dendritic cells [54, 55], each of the anthrax toxin targets either pro-inflammatory cytokines or anti-inflammatory cytokine responses to better counter host innate defenses.

The brisk increase of cAMP induced by EdTx can alter PKA-dependent intracellular signaling. Interestingly, PKA inhibits Mkk4/7, via its effects on Rho and Mek1/2 through alteration of Ras and Raf signaling. In this regard, the mammalian Rho family of GTPases including RhoA, Rac1, and Cdc42 play pivotal roles in

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controlling many cellular functions including cell polarity, motility, proliferation, apoptosis, and cytokinesis. MEK1 and MEK2, also called MAP kinase kinase and ERK activator kinase, are dual-specificity protein kinases that function in a mitogen activated protein kinase cascade controlling both cell growth and differentiation. Therefore, Mkk4/7 and MAPK/ERK kinase (Mek) are potential common targets of both LeTx and EdTx and it is not surprising that EdTx also alters the functions of cells that mediate adaptive immune responses. For example, sublethal doses of EdTx can suppress T cell activation through the inhibition of NFAT (nuclear factor of activated T cells) and AP-1, two transcription factors essential for cytokine secretion [56]. In addition, a recent study has shown that both LeTx and EdTx inhibit chemotaxis of T cells and macrophages by compromising signaling through CXC (by macrophage inflammatory protein 1 alpha or MIP1α) and CC (by Stromal Cell Derived 1 alpha SDF1α) chemokine receptors [35]. It is important to indicate that despite these analogies, EdTx differs from LeTx with regard to inhibition of T cell responses. Thus, T cells from mice intraperitoneally injected with LeTx exhibited lower proliferative and cytokine responses to in vitro stimulation via the TCR. Similar treatment with EdTx only transiently affected T cell responsiveness to TCR stimulation [37]. However, T cells that recovered responsiveness to TCR four days after EdTx produced lower levels of TNF-α, IFN-γ and IL-17 [37], whichis consistent with other studies that showed EdTx preferentially inhibits production of inflammatory cytokines [32].

Mice injected with high doses of EdTx exhibited intestinal fluid accumulation, suggesting that EdTx also could affect immune homeostasis in the mucosal tissues of the gut [57]. Interestingly, while expression of ATR1 on the apical surface of intestinal epithelial cells has been reported, no fluid accumulation was seen after intragastric administration of EdTx (Cormet-Boyaka E, unpublished observation). This observation is consistent with the concept that symptoms of gastrointestinal anthrax are not due to toxins released in the intestinal lumen. It is more likely that toxins reach GALT tissues from the basolateral side after being released in the Peyer's patches by bacteria that germinate in this site after uptake of spores. Alternatively, toxins affect GALT tissues after being released into the general circulation.

While most studies have emphasized the inhibitory effect of EdTx on immune cells, evidence existsthat EdTx upregulates the expression of anthrax toxin receptor by macrophages [58] This mechanism could contribute to disease pathogenesis by increasing in the rate of toxin internalization and indicates that EdTx may provide a positive signal for enhanced rather than impaired functions by target cells. In this regard, the transcription factor cAMP response-element binding protein (CREB) induced by EdTx was found unexpectedly to protect macrophages from LeTx-induced death [42]. The EdTx also stimulates the expression of MHC and costimulatory molecules by antigen presenting cells [32, 59]. In contrast with LeTx which inhibits antibody responses, EdTx was shown to act as an adjuvant following administration through the subcutaneous [60], the nasal [59], or the transcutaneous routes (Duverger A, unpublished observation) and augment antibody responses.

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13.5 New Approaches for Inducing Immunity to Anthrax

Live spores of avirulent strains of *Bacillus anthracis* are being used for vaccination of domestic animals worldwide [61, 62]. Until recently, live spore vaccines were used for dermal vaccination of humans in a number of countries [61, 62, 63]. The injection of anthrax toxin into primates reproduces the pathogenesis of infection with *Bacillus anthracis* and as a result, anthrax is considered an exotoxin-mediated disease [64]. Cell-free PA-based vaccines for humans are used both in the United States [65] and the United Kingdom. These vaccines are aluminum hydroxide anthrax vaccine adsorbed (AVA) supernatants from cultures of *B. anthracis* strain V770-NPI-R [61] or culture filtrates from the Sterne strain [65, 66]. The latter AVA currently is used for military personnel in the USA. These vaccines require a series of six injections over an 18-month period with annual boosters.

Several studies have unveiled a major role for anti-PA antibodies in the protection against anthrax [67, 68, 69]. Thus, passive transfer of polyclonal or monoclonal PA-specific IgG antibodies to mice, rabbits, guinea pigs and monkeys conferred protection against both cutaneous and inhalational forms of anthrax [64, 67]. In vitro, anti-PA antibodies can protect mouse macrophages from toxicity induced by the LeTx and thereby providing the basis for the in vitro macrophage toxicity assay used as a correlate of protection [70, 71]. In addition, other studies suggest that protection against anthrax involves immune responses to other anthrax toxin components. Thus, both anti-PA and anti-LF antibodies were detected in the sera of individuals with histories of clinical anthrax [72]. As we will be discussed below, effort is being dedicated to the development of new anthrax vaccines to be administered by non-invasive routes, including nasal and oral vaccines.

13.5.1 Approaches for the Development of New PA-Based Vaccines

Most experimental anthrax vaccines that either are or have been tested are PA-based and consist of recombinant PA given with various adjuvants [68, 73]. The first evidence that non-invasive nasal immunization may be useful for improving vaccination strategies against anthrax came from the observation that both intranasal and subcutaneous immunizations with PA induced high levels of anti-PA serum IgG antibodies, which could protect J774A.1 cells against LeTx, in vitro [74]. However, only nasally immunized mice contained high titers of serum IgA antibodies [74]. Another study showed that neutralizing anti-PA antibodies could be induced in mucosal secretions [73]. Thus, mice nasally immunized with recombinant PA and cholera toxin or CpG oligodeoxynucleotides, as mucosal adjuvants, developed high plasma PA-specific IgG responses and neutralizing anti-PA IgA antibodies in mucosal secretions [73]. The current BioThrax anthrax vaccine (i.e., AVA) also was tested as a nasal vaccine in mice. Nasal administration of BioThrax elicited robust

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anti-PA serum and mucosal antibody responses against PA [75]. More interestingly, two nasal doses of BioThrax were sufficient to completely protect the susceptible A/J mice against challenge with *Bacillus anthracis* Sterne spores [75].

In addition to adjuvants, new formulations and delivery systems are being tested for inducing both mucosal and systemic immunity by PA-based anthrax vaccines. Thus, a powder formulation administered intranasally to rabbits provided complete protection, whereas a liquid formulation provided only partial protection [76]. These authors also showed that intradermal administration of PA achieved higher seroconversion rates than intramusculular injection, and more efficiently protected rabbits against aerosol challenge with anthrax spores [76]. Water-in-oil nanoemulsions also were tested for nasal immunization of mice and guinea pigs. Intranasal immunization with recombinant PA mixed in a water-in-oil nanoemulsion as an adjuvant, was effective in inducing both serum anti-PA IgG and bronchial anti-PA IgA and IgG antibodies after one or two mucosal administrations [77]. During the last decade, the transcutaneous route has emerged as an alternative means to induce both mucosal and systemic immunity [78]. Transcutaneous immunization of mice with recombinant PA was shown to induce long-term neutralizing antibody titers, which were higher than those obtained with aluminum-adsorbed PA [79]. A powder formulation of PA given by the transcutaneous route also protected rabbits against aerosol challenge with spores, suggesting that protection against inhalational anthrax could be achieved by transcutaneous application of PA-based vaccines [76].

Besides the protein-based PA vaccines discussed above, plasmid DNA and several live recombinant vectors expressing PA were developed for immunization against anthrax. Almost a decade ago, a plasmid DNA encoding PA was injected intramuscularly into mice and was found to induce serum antibodies with protective activity [80]. One approach to improve the immunogenicity of antigens expressed by plasmid DNA vaccines consists in fusion with the VP22 protein of Herpes Simplex Virus type I. Despite the success of this approach with other plasmid DNA, the immune enhancing mechanisms of VP22 remain poorly understood. Therefore, it is rather difficult to explain the fact that immunization with a plasmid DNA expressing PA63 fused to VP22 failed to enhance anti-PA immunity and did not improve the protection of A/J mice against live spore challenge [81]. Recombinant *Salmonella* vectors are well-recognized as an effective system for delivery of heterologous transgenes by multiple routes, including the oral route. In an attempt to develop live recombinant anthrax vaccines, whole PA or portions of this molecule were expressed in *Salmonella* vectors [82, 83, 84]. Intravenous injection of mice with an auxotrophic mutant of *Salmonella typhimurium* expressing the PA gene as a fusion with the signal sequence of the hemolysin (Hly) export system, led to high levels of anti-PA antibodies, which could protect mice against intraperitoneal challenge with lethal doses of *Bacillus anthracis* [83]. Another export system for the expression of heterologous antigens as secreted molecules, the cytolysin A hemolysin of *Salmonella enterica serovar typhi* (ClyA), was used to enhance the immunogenicity of the PA domain 4 expressed in the *Salmonella typhi* vaccine strain CVD 908-htrA [85]. More than 70% of mice immunized intranasally

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with the *Salmonella* vaccine secreting PA domain 4 developed high serum anti-PA antibody responses, while no significant responses were seen in mice that received the live vector expressing PA domain 4 in the cytoplasm [85]. However, the PA antigen expressed by *the Salmonella* vector seems be crucial for the efficacy of these recombinant vectors as oral vaccines. In this regard, *Salmonella typhimurium* vectors, which expressed full-length PA, PA domains 1 and 4, or PA domain 4 fused to the Salmonella typhi ClyA, were produced. Oral immunization of mice with these constructs showed that only the *Salmonella* expressing full-length PA protected mice against aerosol challenge with *Bacillus anthracis* STI spores [84]. The recombinant *Salmonella* expressing PA domains 1 and 4 protected 25% of mice, while the vector expressing PA domain 4 alone afforded no protection [84].

Replication-deficient adenoviruses are proven vectors for nasal and oral delivery of foreign DNA. One such vector, the human adenovirus serotype 5 (Ade5), was engineered to express PA. A single intramuscular injection of mice with the Ade5-PA evoked higher levels of anti-PA antibodies and enhanced protection against *Bacillus anthracis* lethal toxin challenge than the AVA [86]. However, mucosal application of this Ade5-PA has not been reported. An attenuated nontoxinogenic nonencapsulated *Bacillus anthracis* spore vaccine expressing high levels of PA also has been developed and tested in oral immunization studies in animal models. This vaccine induced anti-PA secretory IgA antibodies into the intestinal lumen of orally immunized guinea pigs, indicating that spores of attenuated *Bacillus* strains could be a viable oral vaccine delivery system for induction of mucosal immunity [87].

The use of attenuated bacteria or viruses as vaccine vectors has raised safety concerns. *Lactobacilli* are commensals of the gut and genitourinary tract and thus, generally are regarded as safer than attenuated bacteria for use as delivery vectors for vaccine antigens and other regulatory molecules. Attempts were made to express PA as an intracellular, surface-anchored or extracellular antigen in *Lactobacilli casei* and these recombinant vectors were tested for oral immunization [88]. Plants also could represent a "safer" vaccine vector alternative to attenuated bacteria and viruses. In this regard, PA has been successfully expressed in transgenic tobacco chloroplasts [89, 90]. Subcutaneous immunization of mice with partially purified chloroplast-derived PA yielded anti-PA antibody levels, which protected mice against intraperitoneal challenge with lethal toxin [90]. After the development of a rice-based oral vaccine expressing cholera toxin B subunit [91], one would expect that same strategy could aid the development of a plant-based cold-chain-free oral anthrax vaccine.

13.5.2 Experimental Anthrax Vaccines for Induction of Immunity Against Anthrax Toxin Components and Spores

Immunization with plasmid DNA encoding the LF antigen alone was reported to provide complete protection in mice [92]. This study also showed that co-immunization with PA and LF DNA promoted four to five times greater titers of

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anti-PA and anti-LF antibodies than those induced by the individual DNA plasmid given alone. The PA binding sites of anthrax lethal factor and edema factor are highly homologous, and are both located in the N-terminal fragment of these molecules. An attenuated adenovirus encoding the N-terminal region 1–254 amino acids of EF was constructed [93]. After three intramuscular immunizations, this vaccine could protect approximately 50% of mice from subcutaneous challenge with 100 × LD50 of *Bacillus anthracis* spores [93]. The study also demonstrated that anti-EF antibodies cross-react with anthrax lethal factor and can neutralize the activity of both LeTx and EdTx [93].

Other investigators addressed the question of whether or not protection against *Bacillus anthracis* infection can be improved by inducing antibodies that target anthrax toxin components and spores or the capsule. In fact, the addition of formaldehyde-inactivated spores of *Bacillus anthracis* to PA could elicit total protection against challenge with virulent *Bacillus anthracis* strains in mice and guinea pigs [94]. Other studies now have demonstrated that the *Bacillus* collagen-like surface protein of anthracis (BclA), which is located on the surface of exosporium could substitute whole spores in vaccine formulations. For example, mice immunized with the combination of PA- and BclA-encoding plasmid DNA better survived challenge with *Bacillus anthracis* Ames spores than those immunized with PA- or BclA-encoding plasmids alone [95]. In another study of mice primed two weeks earlier with suboptimal doses of PA, injection of a non-glycosylated recombinant BclA PA protected mice against lethal challenge with *Bacillus anthracis* Stern spores [96]. Components of the capsule also were reported to improve anthrax immunity. Thus, nasal immunization of rabbits with a vaccine consisting of PA conjugated to a 10-mer peptide of the poly-D-glutamic acid capsule of *Bacillus anthracis* was found to provide superior protection against *Bacillus anthracis* infection than PA alone [97].

The combination of PA plus the N-terminal 1–254 fragment of LF (LFn^{1-254}) has been used as a "molecular syringe" to introduce foreign antigens coupled to LFn^{1-254} into target cells. This strategy successfully delivered short peptides [98, 99] as well as proteins [100, 101]. Mechanistic studies with PA mutants attenuated in self-assembly or translocation [98, 102] or cells with disrupted furin genes [103] showed that delivery of Ag involves the same toxin self-assembly and translocation steps that occurr during intoxication. Most interestingly, systemic injection of mice with PA in association with EF was found to enhance the levels of serum anti-PA antibody responses above those achieved by injection of PA alone [60]. The adjuvant activity of EdTX for anti-PA immunity was confirmed after nasal immunization with a mutant of EdTx consisting of a mutant EF with reduced adenylyl cyclase activity [59]. It is important to note that EdTx as a nasal adjuvant also induced neutralizing anti-PA antibodies in mucosal secretions [59]. Furthermore, high anti-EF antibodies were induced after nasal immunization with EdTx (Duverger A, unpublished observation). Both native and mutant EdTx were shown to upregulate the expression of costimulatory molecules by antigen presenting cells [32, 59], and this certainly accounts in the adjuvant activity of these molecules.

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13.6 Summary and Perspectives

The recent cases of inhalation anthrax in the US, as well as the threat of other deliberate dissemination of anthrax spores as agents of bio-warfare has spurred increased anthrax research since 2001. A major result of this burst is a greater appreciation of mucosal tissues of the gastrointestinal and respiratory tract as both portals of entry of *Bacillus anthracis* spores and initial sites of interaction of host immune cells with *Bacillus anthracis* pathogenesis factors. Consistent with this new understanding, strategies are being developed for potential induction of both mucosal and systemic immunity to *Bacillus anthracis* toxins and other spore-related pathogenesis factors (Table 13.1). The recent identification of EdTx derivatives as adjuvants for induction of high levels of immunity against PA and EF, as well as co-administered unrelated antigens [59], could provide a unique platform for the development of multivalent mucosal anthrax vaccines.

Table 13.1 Summary of new approaches investigated for induction of protective immunity against inhalational and gastrointestinal anthrax[a]

Antigens	Formulations	Delivery Routes
PA (Recombinant protein)	In saline with CpG or cholera toxin derivatives as adjuvants	Nasal
	With alum	Nasal
	Powder	Nasal
	Water-in-oil nanoemulsion	Nasal
	Powder	Transcutaneous
PA (DNA)	Plasmid DNA	Injection sc or im
	Salmonella vector	Nasal
	Salmonella vector	Oral
	Adenoviral vector	Injection im
	Lactobacilli vector	Oral
	Transgenic plants	Injection sc
EF (DNA)	Plasmid DNA	Injection
PA (DNA) and EF (DNA)	Plasmid DNA	Injection
PA (DNA) and BclA (DNA)	Plasmid DNA	Injection Injection
Native PA plus native or mutant EF (Recombinant proteins)	In saline	Nasal Transcutaneous Oral
Recombinant PA conjugated to a 10-mer peptide of the poly-D-glutamic acid capsule	In saline	Nasal

[a]While most current vaccines are given by intramuscular or subcutaneous injection, the new approaches recently investigated for induction of anti-anthrax immunity involved (or could be adapted for) delivery by routes (i.e., oral, nasal or transcutaneous) were more likely to induce both mucosal and systemic immunity. Sc: subcutaneous; im: intra-muscular.

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