Enterotoxin Adjuvant for mucosal immunity

Masafumi Yamamoto¹, Hiroshi Kiyono²

¹Department of Oral Medicine, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-Nishi, Matsudo 271-8587
²Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639

Correspondence to:
Dr. Masafumi Yamamoto
E-mail: fumi@mascat.nihon-u.ac.jp

Abstract

The basic structure and biological function of cholera toxin (CT) and heat-labile toxin of enterotoxigenic Escherichia coli (LT), which induces diarrhea in humans, are similar. Both CT and LT act as adjuvants for the enhancement of mucosal and serum antibody responses to the mucosally co-administered protein antigen. CT acts as an adjuvant by inducing IL-4-dependent Th2 cytokine responses, which provide help for antigen-specific secretory IgA (S-IgA) as well as serum IgG1, IgA and IgE antibody responses. On the other hand, LT induces Th1- and partly IL-4-independent Th2-type cells with subsequent serum IgG1, IgG2a and mucosal S-IgA responses. Nontoxic mutant CT (mCT) and the chimeric molecule that combine the A subunit of mCT with the B subunit of LT (mCTA/LTB), like nCT, act as mucosal adjuvants and induce mucosal IgA and systemic IgG and IgA antibody responses. These studies indicate that ADP-ribosyltransferase activity can be separated from the adjuvant properties of CT and LT. Further, those nontoxic enterotoxin derivatives should be considered as candidate mucosal adjuvants for vaccinating humans.

Key Words:
mucosal immunity, cholera toxin, heat-labile toxin, mucosal adjuvant

Introduction

The mucosal surface areas where most pathogens enter the host are protected by antibodies of the secretory IgA (S-IgA). This isotype constitutes more than 80% of all antibodies produced in mucosa-associated tissues, and S-IgA antibodies are induced, transported, and regulated by a unique mechanism of mucosal intranet consisting of Th1/Th2 cells, IgA committed B cells, dendritic cells and epithelial cells that is completely different from those involved in systemic antibody responses (1). The mucosal immune system is an integrated network of tissues, cells and effector molecules, which protects the host from environmental pathogens and antigens. Mucosal lymphocytes exhibit unique homing receptors, integrins and chemokine receptors, which recognize ligands expressed on mucosal endothelial cells, allowing their specific migration to and retention in mucosal tissues for the induction and regulation of antigen-specific S-IgA in external secretions (1). Thus, the mucosal immune system is separate from the peripheral lymphoid tissues providing immune protection for internal organs and tissues. Therefore, the induction of peripheral immune responses by parental immunization does not result in significant mucosal immunity (1). In contrast, mucosal immunization results in the generation of protective immunity in external secretions and in the peripheral compartment as well. For the control of infectious diseases, mucosal vaccination becomes an attractive approach since it possesses a capability of maximally generating antigen-specific immune responses at both external and internal compartments of the host. To accomplish this goal, different forms of mucosal adjuvant, vector, and delivery system are being investigated for their ability to induce well-defined immune responses to protect individuals from mucosal pathogens (1). In this regard, cholera toxin (CT) is an effective mucosal adjuvant that can support the induction of antigen-specific mucosal and systemic immune responses against coadministered protein antigen (2-7). In this article, we will summarize our recent progress in the characterization of molecular and cellular mechanisms for mucosal adjuvant activity of CT in comparison to its related heat labile toxin (LT) of enterotoxigenic Escherichia coli (ETEC). Further, we will briefly summarize the development of mutant derivatives of CT and LT as novel nontoxic mucosal adjuvants.
The mucosal immune system

The mucosal immune system is anatomically and functionally divided into sites where foreign antigens are encountered and selectively taken up for initiation of immune responses and the more diffuse collections of B and T cells, plasma cells, and antigen-presenting cells, which comprise the effector cells for induction and regulation of mucosal immunity. Collectively, these tissues associated with this activity are known as inductive sites. In case of the intestinal tract, Peyer’s patches are the most well characterized tissue which represent gut-associated lymphoreticular tissue (GALT), a major inductive site for orally administered antigens (1). On the other hand, the major inductive sites for nasal/inhaled antigens appear to be the palatine tonsils and adenoids, which together form a physical barrier of lymphoid tissues termed the Waldeyer’s ring, now more frequently referred to as a nasopharyngeal-associated lymphoreticular tissue (NALT) (1).

Peyer’s patches, a well characterized GALT, have been shown to be the major inductive sites for the initiation of S-IgA antibody responses in the gastrointestinal tract. The subepithelial dome region of Peyer’s patches contain antigen-presenting cells of all major types including dendritic cells, macrophages and major histocompatibility complex (MHC) class II+ B cells for processing and presentation of appropriate peptides to initiate mucosal immune responses. In addition, germinal centers are located under the dome area and consist of high frequencies of IgA committed or surface IgA+ B cells. Internodular zones are predominantly populated by T cells of both CD4+ and CD8+ subsets for the formation of an effector T cell network consisting of helper T cells and cytotoxic T lymphocytes (CTLs) (1). The antigen-specific T and B cells leave the Peyer’s patches via the efferent lymphatics and are transported to the systemic circulation through the thoracic duct. Finally, these cells are disseminated in mucosal effector sites, e.g., the lamina propria region of the small intestine for the formation of the mucosal intranet with epithelial cells to generate S-IgA antibodies (1) (Fig. 1).

Th cells segregate into two major groups according to the cytokines they secrete. Th1 cells secrete mainly interleukin (IL)-2, interferon (IFN)-γ, and lymphotoxin-α.
and stimulate cell-mediated immunity and cytotoxicity while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which are mainly involved in antibody responses (8). At the systemic level, Th1- and Th2-type immune responses are associated with different patterns of antibody isotypes and subclasses. Thus, complement-fixing antibodies of the murine IgG2a are associated with Th1-type IFN-γ driven immune responses while noncomplement-fixing IgG1 is associated with Th2-type responses and IL-4 production (8, 9). Further, the outcomes of these distinct cytokine patterns are influenced by the unique microenvironment in mucosa-associated tissues. Studies from our group and by others have shown that two Th2 cell-derived cytokines, for example, IL-5 and IL-6, are of particular importance for inducing surface IgA+ (sIgA+) B cells to differentiate into IgA-plasma cells (10-13). Recent studies have shown that non Th2 cell-associated cytokine such as IL-15 supported the differentiation of sIgA+ B cells to IgA plasma cells (14). Further, a group of intestinal sIgA+ B cells associating with B-1 lineage expressed IL-15Rα together with IL-5R. In contrast, B-2 lineage of sIgA+ B cells responded to IL-5 and IL-6 but not IL-15 (14). These findings suggest that two groups of sIgA+ B cells including B-1 and B-2 are the sources for the generation of S-IgA antibodies in external secretions. Further, these two subsets of sIgA+ B cell are regulated by two distinct cytokines, IL-15 and IL-6, together with a common cytokine IL-5.

**CT and LT as mucosal adjuvants**

The basic structure and biological function of CT and LT, which induces diarrhea in humans, are similar; however, the infection caused by the former molecule is associated with a more severe, cramping diarrhea (15-17). The nucleotide sequences of the two toxins are approximately 80% homologous (18, 19). Both CT and LT consist of an enzymatically active A subunit separated from the plane of a ring formed by five smaller, identical B subunits (20, 21). The A subunit is composed of a globular structure linked to the B subunit by a trypsin-sensitive loop and a long α helix, the C-terminus, which enters into the central cavity of the B subunit, thus anchoring the A subunit to the B pentamer (21). The A subunit contains an ADP-ribosyltransferase active pocket that binds nicotinamide adenine dinucleotide (NAD) and catalyzes ADP ribosylation of Gsα (22). This GTP binding protein activates adenyyl cyclase with subsequent elevation of cAMP, which in epithelial cells results in secretion of water and chloride ions into the small intestine (23). On the other hand, the B pentamer is arranged in a cylinderlike structure, with a central cavity that exposes five symmetrical cavities that are responsible for binding to cell surface receptors (21). The receptor binding site is specific for a variety of galactose containing molecules and shows a different fine specificity between CT and LT. The B pentamer of CT (CT-B) selectively binds GM1, whereas LT-B is ligand for GM1, GM2 and asialo GM1 cell surface receptors (24-26). The different receptor binding activities of the CT and LT may promote different adjuvant properties that are exhibited by two molecules, as will be discussed below.

CT and LT are potent immunogens and induce antigen-specific S-IgA and serum IgG and IgA antibody responses (27-29). Furthermore, both toxins can act as adjuvants for the enhancement of mucosal and serum antibody responses to mucosally co-administered protein antigen (2-7, 30, 31). CT acts as adjuvant by inducing CD4+ Th2 cells secreting EL-4, IL-5, IL-6 and IL-10, which provide help for antigen-specific S-IgA as well as serum IgGl, IgA and IgE antibody responses (4, 7). CT has also been shown to selectively inhibit proliferation of CD4+ Th1, but not of Th2, cell lines stimulated by anti-CD3 monoclonal antibody or by PMA and ionomycin (32). Further, CT facilitated B cell switching to IgA and increased the effects of IL-4 and IL-5 on IgG and IgA synthesis in LPS-triggered splenic B cell cultures (33, 34). In addition to these unique immunological characteristic of CT shown by the murine studies, it was also shown that nasal immunization of rhesus macaques with p55gag of simian immunodeficiency virus (SIV) plus CT elicit SIV-specific S-IgA antibody and CTL responses in both mucosal and systemic compartments (35). These additional immunological features of CT further provide supportive evidence for potential usage of the enterotoxin as mucosal adjuvant if one could genetically separate toxicity and immune enhancing activity.

Like the related CT, LT has been widely used for studies of mucosal immunity (2, 30, 36-38). Mucosal immunization of mice with Ag and LT induced mixed CD4+ Th1- and Th2-type cells with subsequent mucosal S-IgA antibody responses (30). Further, our previous study has shown that
LT promotes mucosal IgA antibody responses by an IL-4-independent mechanism (39). In contrast, it was shown that CT provides its adjuvant activity via the IL-4 dependent manner (6,7,39). Since LT differ from CT in terms of the type of immune responses (CT for Th2 while LT for both Th1 and Th2), we can select most suitable mucosal adjuvant for each vaccine based on the nature of infectious agents. In summary, several lines of evidence indicate that CT and LT as mucosal adjuvant are capable of inducing both humoral and cell-mediated immunity in systemic and mucosal compartments.

**Mechanisms for adjuvant effect of enterotoxins**

CD28 on T cells provides a potent co-stimulatory signal following interactions with B7-1 (CD80) and B7-2 (CD86) expressed on antigen-presenting cells (40). With regard to the involvement of CD28/B7 signaling in the adjuvanticity of CT, it has been reported that both native CT and a fusion protein with the intact A1 subunit enhanced B7-1 and B7-2 expression on B cells (41). In contrast, other studies have shown that CT enhances B7-2, but not B7-1, expression by macrophages and administration of anti-B7-2 monoclonal antibodies inhibited keyhole limpet hemocyanin (KLH)-specific serum IgG and mucosal IgA antibody responses in mice given KLH plus CT orally (42). Our studies have shown that both CT and LT enhances B7-2 expression on B cells and macrophages in Peyer’s patches (39). Further, addition of LT- or CT-treated antigen-presenting cells to anti-CD3 triggered CD4+ T cells resulted in the induction of T cell proliferative responses. Moreover, these responses were inhibited by monoclonal antibodies to B7-2 (39), implying that the CD28/B7-2 signaling pathway is of major importance for the adjuvant activity of both enterotoxins (Fig. 2). Interestingly, most recent studies have shown that oral immunization of CTLA4-H1 transgenic mice with CT as adjuvant failed to induce antigen-specific antibody responses in both mucosal and systemic compartment. On the other hand, CD28+ mice developed near normal gut mucosal IgA responses but poor serum antibody responses (43). These results suggest that alternative costimulatory pathways in addition to CD28/B7-2 are involved for the induction of adjuvant activity of CT.

Both CT and LT can directly affect T cells; however, LT differs from CT in terms of the regulation of Th1 and Th2 cell responses. CT inhibits proliferative responses and cytokine synthesis of Th1 clones, but had no effect on Th2

![Fig.2. Mechanisms of mucosal adjuvanticity of CT and LT.](image-url)
clones activated by TCR-mediated signaling (32). Our studies have also shown that CT directly affects Peyer's patch CD4+ T cells activated via the TCR-CD3 complex with selective inhibition of Th1 responses (39, 44). As a result, Th2-type responses are dominated under the influence of CT. However, LT directly affected activated CD4+ T cells and inhibited interleukin (IL)-4 synthesis in normal (IL-4+/+) mice and maintained IL-5 production in IL-4 deficient (IL-4−/−) mice while CT preferentially inhibited Th1-type cytokines in IL-4−/− mice and did not support IL-5 production in IL-4−/− mice (39). In support of this, oral immunization of IL-4−/− mice with protein antigen plus LT, but not CT, as a mucosal adjuvant induced antigen-specific Th2-type cytokines including IL-5, IL-6 and IL-10 with subsequent S-IgA antibody responses in the intestinal mucosal areas (6, 39). These findings suggest that the initial event induced by CT in CD4+ T cells involves up-regulation of IL-4, and this precedes other Th2 cytokine activation events, whereas LT affects CD4+ T cells and support IL-4-independent signal transduction pathway for induction of other Th2 cytokines (Fig. 2).

**Nontoxic mutant enterotoxins as a new generation mucosal adjuvant**

Despite the potent mucosal adjuvant activity of native (n) CT and nLT, both enterotoxins cause severe diarrhea and are thus unsuitable for use in humans (28). Therefore, a number of nontoxic mutant derivatives of CT or LT have been constructed (36, 45-50). We have also generated mutant (m) CT by substituting a single amino acid in the ADP-ribosyltransferase active center of the A subunit and have created two mutants of CT (S61F and E112K) (52)(Fig. 3). These two gene manipulated forms of mCT did not induce ADP-ribosylation, cAMP formation, or fluid accumulation in ligated ileal loops and thus are nontoxic (51). Importantly, these mCTs still supported antigen-specific immune responses when co-administered parenterally with protein antigen (51). Further, we have demonstrated that mCT acts as a mucosal adjuvant by inducing CD4+ Th2 cells secreting IL-4, IL-5, IL-6 and IL-10, which provided effective help for antigen-specific mucosal S-IgA, as well as serum IgG1, IgE and IgA antibody responses (31). Mucosal adjuvant activity of mCT S61F and E112K has been demonstrated using several antigens including ovalbumin, tetanus toxoid, *Streptococcus pneumoniae* and influenza virus (5, 31). Further, nasal immunization with surface protein of *Streptococcus mutans* with mCT E112K elicited antigen-specific IgM, IgG and IgA in serum and IgA antibody responses in saliva (52). These findings provide evidence that ADP-ribosyltransferase activity can be separated from the adjuvant properties of CT. In our most recent study, we have constructed a novel nontoxic form of chimeric mucosal adjuvant that combines the A subunit of mCT E112K with the B subunit of LT (mCTA/LTB) (53). Nasal immunization of mice with hemagglutinin (HA) vaccines plus mCTA/LTB elicited significant HA-specific IgA antibody responses in mucosal compartments, which provided protective immunity against influenza virus infection. Further, nasal vaccination with tetanus toxoid (TT) and mCTA/LTB resulted in the induction of protective immunity in the systemic compartment. These findings demonstrated that the nontoxic chimera molecule of mCTA/LTB can support IgA and IgG mediated protective immune responses in mucosal and systemic compartments, respectively. Importantly, while TT plus nCT induce high antigen-specific IgE responses, use of the chimera molecule as mucosal adjuvant did not (53). These findings indicate that mCTA/LTB is an effective and safe mucosal adjuvant for the induction of protective immunity in both mucosal and systemic compartments.
References
29. Takahashi I, Kiyono H, Jackson RJ, Fujihashi K, Staats HF, ...


