

Review

Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA

Jan Holmgren^{a,*}, Jenni Adamsson^a, Fabienne Anjuère^c, John Clemens^b, Cecil Czerkinsky^c, Kristina Eriksson^a, Carl-Fredrik Flach^a, Annie George-Chandy^a, Ali M. Harandi^a, Michael Lebens^a, Thomas Lehner^d, Marianne Lindblad^a, Erik Nygren^a, Sukanya Raghavan^a, Joaquin Sanchez^a, Michael Stanford^d, Jia-Bin Sun^a, Ann-Mari Svennerholm^a, Sara Tengvall^a

^a Department of Medical Microbiology & Immunology and Göteborg University Vaccine Research Institute (GUVAX), Göteborg University, P.O. Box 435, SE-40530 Göteborg, Sweden

^b International Vaccine Institute, IVI, Seoul, Republic of Korea

^c I.N.S.E.R.M. UNITE 364, Faculté de Médecine-Pasteur, Avenue de Vallombrose, 06107 Nice Cedex 02, France

^d Guy's London School of Medicine, London University, UK

Received 5 October 2004; received in revised form 27 October 2004; accepted 7 November 2004

Available online 7 December 2004

Abstract

Mucosal immunisation may be used both to protect the mucosal surfaces against infections and as a means for immunological treatment of peripheral immunopathological disorders through the induction of systemic antigen-specific tolerance ('oral tolerance'). The development of mucosal vaccines, whether for prevention of infectious diseases or for oral tolerance immunotherapy, requires efficient antigen delivery and adjuvant systems that can help to present the appropriate vaccine or immunotherapy antigens to the mucosal immune system. The most potent (but also toxic) mucosal adjuvants are cholera toxin (CT) and the closely related *Escherichia coli* heat-labile enterotoxin (LT), and much effort and significant progress have been made recently to generate toxicologically acceptable derivatives of these toxins with retained adjuvant activity. Among these are the non-toxic, recombinantly produced cholera toxin B-subunit (CTB). CTB is a specific protective antigen component of a widely registered oral cholera vaccine as well as a promising vector for either giving rise to mucosal anti-infective immunity or for inducing peripheral anti-inflammatory tolerance to chemically or genetically linked foreign antigens administered mucosally. CT and CTB have also recently been used as combined vectors and adjuvants for markedly promoting ex vivo dendritic cell (DC) vaccination with different antigens and also steering the immune response to the in vivo-reinfused DCs towards either broad Th1 + Th2 + CTL immunity (CT) or Th2 or tolerance (CTB). Another type of mucosal adjuvants is represented by bacterial DNA or synthetic oligodeoxynucleotides containing CpG-motifs, which especially when linked to CTB have been found to effectively stimulate both innate and adaptive mucosal immune responses. The properties and clinical potential of these different classes of adjuvants are being discussed.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Mucosal adjuvants; Anti-infection; Anti-immunopathology

Contents

1. Introduction	182
2. Oral cholera B subunit—whole cell (CTB–WC) cholera vaccine	183

* Corresponding author.

E-mail address: jan.holmgren@microbio.gu.se (J. Holmgren).

3.	CT and LT and derivatives as mucosal adjuvants	184
3.1.	The adjuvant action of CT and LT	184
3.2.	Development of non-toxic derivatives as mucosal adjuvants	184
4.	CT and CTB as adjuvants for dendritic cell vaccination	185
5.	CTB::CpG—a novel potent immuno-modulating adjuvant	185
6.	Use of CTB for mucosal immunotherapy with proof-of-principle clinical study in patients with Behçet's disease	187
	References	187

1. Introduction

Mucosal immunisation has for good reasons attracted much interest, mainly as a means to induce protective immunity against infections but also as a possible approach towards specific immunotherapy in conditions caused by harmful immune responses to autoantigens or selected foreign antigens resulting in tissue-damage (Fig. 1) [1–3].

The majority of infectious agents use a mucosal surface as their entry point for infection. In these infections topical application of a vaccine at the mucosal surface is often needed to induce a protective immune response. Several gastrointestinal infections caused by e.g. *Helicobacter pylori*, *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC), *Shigella* spp., *Clostridium difficile*, rotaviruses and calici viruses are examples of such infections. Similarly, respiratory infections caused by e.g. *Mycoplasma pneumoniae*, influenza virus and respiratory syncytial virus, and genital infections caused by e.g. HIV, *Chlamydia*, *Neisseria gonorrhoeae* and herpes sim-

plex virus are other examples of infections where a mucosal route of vaccination may be more effective than parenteral vaccination. Collectively, these infections represent an enormous challenge for vaccine development.

There are also important logistic reasons that have made mucosal immunization attractive for public health use, especially in developing countries. Mucosal vaccines, especially oral vaccines, should be easier to administer than parenteral vaccines and also have reduced risk of transmitting the types of infection that are still sadly enough associated with the use of injectable vaccines in several parts of the world, such as hepatitis B virus and HIV infections. Mucosal vaccines should also in many settings have greater acceptability than injectable vaccines.

Much less well appreciated than vaccination against infections is that mucosal immunisation may also be used to induce peripheral tolerance to prevent or suppress the development of harmful immune responses to ingested or inhaled foreign proteins. This phenomenon, often referred to as 'oral tolerance', because it was initially documented by the effect of oral administration of antigen, is characterized in that an individual who has ingested or inhaled an antigen may become refractory or have diminished capability to develop an immune response when re-exposed to the same antigen introduced by a parenteral injection. Oral tolerance is an important natural physiological mechanism to avoid development of T cell mediated delayed type hypersensitivity and other allergic reactions to ingested food proteins and other antigens in case these antigens would reach the body interior in an undegraded form. Since oral tolerance, at variance with many other forms of immunotherapy, is antigen specific, the induction of oral tolerance has become an attractive strategy for prevention and potential treatment of illnesses that are caused by immunopathological reactions against specific antigens encountered or expressed in preferentially non-mucosal tissues including both foreign antigens and autoantigens [1–3].

Although oral tolerance and induction of mucosal immunity in response to a mucosal immunisation usually involve different types of antigens, they are, however, not mutually exclusive aspects of an immune response. Oral tolerance is the default response to ingested food proteins or autoanti-

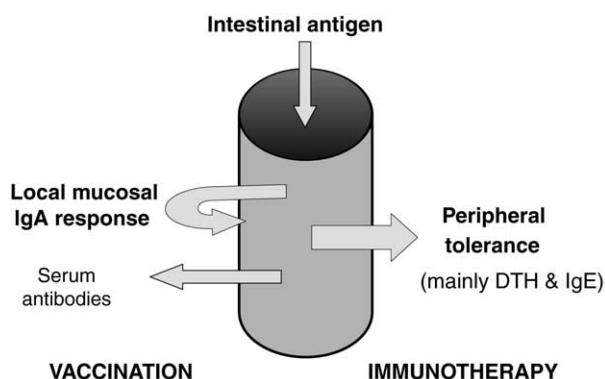


Fig. 1. Possible outcomes of mucosal immunization: a local mucosal immune response and/or peripheral tolerance. The mucosal immune response may comprise either or both of secretory IgA antibodies and T cell mediated immunity, whereas the peripheral immunological tolerance ('oral tolerance' when induced by an orally administered antigen) mainly comprises different types of regulatory T cells, which can suppress systemic immune responses including allergic responses to ingested or inhaled undegraded antigens. Important practical applications of mucosal immunization include both the development of vaccines that can protect against infection and although as yet less advanced in their development, vaccine formulations for immunotherapy of, for instance, autoimmune and allergic disorders.

gens and is difficult or impossible to induce with strong immunogens, whilst the converse is true for a mucosal immune response. However, sometimes the same mucosal immunisation may concomitantly give rise both to a significant IgA antibody response locally and to tolerance or suppressed immune responsiveness peripherally. Indeed, certain cytokines, such as transforming growth factor (TGF)- β and interleukin (IL)-4, are known to be involved both in the development of the mucosal immune response associated with local production of secretory IgA at the mucosal surface and in the induction of oral tolerance peripherally.

However, despite the many attractive features of mucosal immunization it has often proved difficult in practice to stimulate strong mucosal IgA immune responses and protection by oral-mucosal administration of antigens, and the results to date of mucosal vaccination efforts using soluble protein antigens have, with a few notable exceptions, been rather disappointing. Indeed, only half a dozen of the vaccines that are currently approved for human use are administered mucosally: the oral polio vaccine, two types of oral vaccines against cholera (composed of either killed whole-cell cholera vibrios plus CTB or of a live-attenuated cholera vaccine strain), an oral live-attenuated typhoid vaccine, an oral adenovirus vaccine (restricted to military personnel), an oral BCG strain used in Brazil for vaccination against tuberculosis, and since July 2004, also an oral rotavirus vaccine initially licensed in Mexico. At the same time, two recent additional mucosal vaccines, an oral live-attenuated vaccine against rotavirus diarrhoea and a nasal enterotoxin-adjuvanted inactivated influenza vaccine, were withdrawn after a short time on the market because of potential serious adverse reactions (intussusception and facial palsy, respectively), thus illustrating the complexity of mucosal vaccine development. Likewise, in the area of oral tolerance, although promising results were obtained in initial clinical trials that used this principle for immunotherapy of autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, larger randomised placebo-controlled multi-center trials failed to show any significant therapeutic benefits of these agents over those achieved by the placebo [3].

To overcome the obstacles to the development of a broader range of mucosal vaccines and mucosal immunotherapies, there is a need to find more efficient means of delivering the appropriate antigens to the mucosal immune system, and/or to develop effective, safe mucosal adjuvants or immunoregulatory agents that, depending on the goal and the systems used, will provide protective immunity against infectious agents or induce suppression of peripheral immunopathological disorders, respectively. In this brief review, we will describe some recent advances made with the oral cholera vaccine and discuss the two potentially most promising classes of mucosal antigen-delivery and adjuvant systems available for either or both of inducing mucosal immunity and systemic tolerance. The first group comprises cholera toxin (CT) and the closely analogous heat-labile enterotoxin (LT) produced

by certain strains of *Escherichia coli*, and different detoxified derivatives of CT and LT including their completely non-toxic B subunit components (CTB or LTB) (Fig. 2). The other group of recently described mucosal adjuvants to be discussed is represented by bacterial DNA or synthetic oligodeoxynucleotides containing CpG-motifs, especially as linked to CTB, which through binding to Toll-like receptor 9 (TLR9) on mucosal antigen-presenting cells (APC) can effectively stimulate both innate and adaptive immune responses.

2. Oral cholera B subunit—whole cell (CTB–WC) cholera vaccine

As mentioned, CTB is a protective antigen component together with inactivated whole cell *Vibrio cholerae* bacteria in a widely registered (ca. 50 countries world-wide) oral cholera vaccine (Dukoral®), thus representing one of very few mucosal vaccines available for human use [4,5]. Many studies have shown that the CTB–WC vaccine is safe and immunogenic in both children and adults in cholera endemic as well as non-endemic countries, stimulating gut mucosal immune responses to the CTB and WC vaccine components. A good protective efficacy of the vaccine has been demonstrated, being 85% for the first 6 months after vaccination in both endemic and non-endemic populations, and remaining at or above 60% for at least 2 years in vaccinated adults and in children above age 5 years. In children below age 5, the short-term efficacy is very high (100% for the first 6 months when tested in a field trial in Bangladesh), but of shorter duration than in older children and adults. Protection is mediated by locally produced IgA anti-bacterial and anti-toxic antibodies blocking bacterial colonization and cholera toxin action, respectively, for longer term protection mucosal memory for rapid induction of mainly anti-colonisation IgA antibodies is important since these antibodies can then block bacterial colonization and multiplication at the intestinal surface before the infection has progressed to cause disease [5].

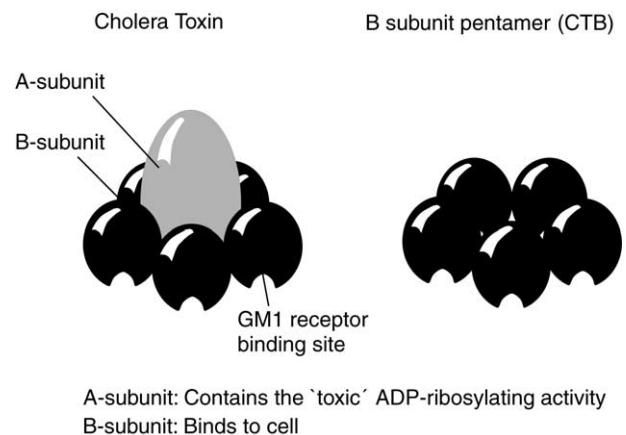


Fig. 2. Molecular model of cholera toxin and its B subunit pentamer.

The CTB–WC cholera vaccine also offers significant short-term cross-protection (through the CTB component) against diarrhea caused by LT-producing enterotoxigenic *E. coli* (ETEC), for which there is currently no other vaccine available [5]. The CTB–WC cholera vaccine has recently also been shown to give rise to substantial herd immunity (Clemens et al., to be published) and has been found to substantially reduce overall diarrhea morbidity in areas where cholera and ETEC-diarrheas are common. A large effectiveness study to assess the role of the vaccine in public health control programs against cholera in Africa has recently also been undertaken. Results indicate that protection even under these conditions and in a population where ca. one-third may be HIV-infected is similar to the efficacy demonstrated in the previously cited phase three efficacy trials, being ca. 80% against cholera as presented to the local treatment centers and near 90% against severe cholera disease associated with dehydration (Clemens, personal communication).

A second type of oral cholera vaccine, based on a live attenuated *V. cholerae* strain deleted in its cholera toxin gene from the ability to make the toxic-active A subunit, is also registered in some countries for use in travelers. However, different from the CTB–WC vaccine the live, attenuated cholera vaccine failed to generate significant protection when tested in a large field trial in Indonesia, and thus is not recommended by WHO for use in cholera endemic countries [4].

3. CT and LT and derivatives as mucosal adjuvants

Besides being strong mucosal immunogens, both CT and LT are powerful mucosal adjuvants. They strongly potentiate the immunogenicity of most other antigens, whether these are linked to or simply admixed with the toxins, provided that the other antigen is given at the same time and at the same mucosal surface as the toxins. However, since both CT and LT are normally considered to be much too toxic for human use, great efforts have recently been made to define whether it would be possible to separate the adjuvant and toxic activities as a basis for development of mucosal adjuvants for human use.

3.1. The adjuvant action of CT and LT

CT and LT can affect several steps in the induction of a mucosal immune response. These effects, which alone or in combination might explain their strong adjuvant action after oral immunisation, include: (1) increased permeability of the intestinal epithelium leading to enhanced uptake of co-administered antigen, (2) enhanced antigen presentation by various APCs, (3) promotion of isotype differentiation in B cells leading to increased IgA formation, and (4) complex stimulatory as well as inhibitory effects on T cell proliferation and cytokine production. In addition, CT and LT have been shown to not only avoid to induce oral tolerance but also to

abrogate otherwise efficient regimens for tolerance induction by oral antigen administration [6–8].

Among these many effects, those leading to enhance antigen presentation by various APC are probably of the greatest importance for the adjuvant activity. CT markedly increases antigen presentation by dendritic cells (DC), macrophages and B cells and has also been found, at least in vitro, to make intestinal epithelial cells to become effective APC. Consistent with this activity, CT up-regulates the expression of MHC/HLA-DR molecules, CD80/B7.1 and CD86/B7.2 co-stimulatory molecules as well as chemokine receptors such as CCR7 and CXCR4 on both murine and human DC and other APC [9,10]. Importantly, CT also induces the secretion of IL-1 β from DC [10] thus supporting similar observations made earlier for macrophages. IL-1 not only induces the maturation of DC, but is also by itself an efficient mucosal adjuvant when co-administered with protein antigens and might mediate a significant part of CTs adjuvant activity.

It has been claimed that CT primarily induces Th2 type immune responses characterized by CD4⁺ T cells producing IL-4, IL-5, IL-6 and IL-10 and by the production of IgA, IgG1 and IgE antibodies. LT, on the other hand, has been reported to induce a mixed Th1 and Th2 types immune response. However, other studies have shown that also CT can induce mixed Th1 and Th2 type of immune responses, in contrast to CTB, which appears to induce a more restricted Th2 type of immune response [10]. Thus DC, which had been pre-treated in vitro with a protein antigen (ovalbumin, OVA) linked to or admixed with CT and then injected into mice, in an antigen-specific manner induced both Th1 and CTL responses in addition to a Th2 response. In contrast, DC pulsed in vitro with OVA linked to CTB only gave rise to a Th2 type of immune response.

3.2. Development of non-toxic derivatives as mucosal adjuvants

To avoid toxicity, isolated CTB and LTB have been explored for their ability to augment immune responses also against co-administered antigens. However, their capacity as mucosal adjuvants has proved to be much less than that of the holotoxins. Indeed, both CTB and LTB are poor adjuvants when given to animals together with non-coupled antigens by the oral route, although they display a more significant adjuvant activity when administered via the nasal route. Adjuvanticity of CTB or LTB is much improved when coupled to antigens, due to the increased uptake of coupled antigen across the mucosal barrier and its more efficient presentation coupled antigen not only by DC and macrophages but also by naïve B cells.

Recently, site-directed mutagenesis has permitted the generation of LT and CT mutants that have reduced toxicity, but which retain significant adjuvanticity when given to animals by the nasal-mucosal route or, even though they then perform less well, by the oral-mucosal route [8] (Fig. 3). Two such mutants, LTK63 and LTK72, are currently clinically evaluated

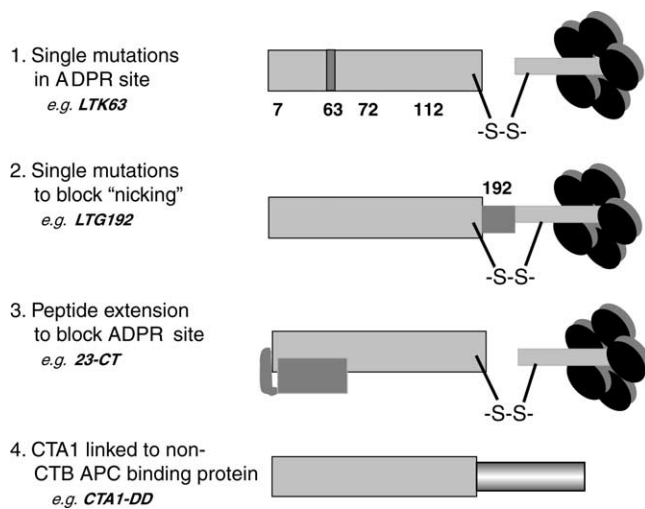


Fig. 3. Examples of mucosal adjuvant molecules based on detoxified cholera toxin or *E. coli* heat labile enterotoxin.

as adjuvants for intranasally administered influenza vaccine (Podda et al., to be published).

To achieve detoxification of CT, another type of mutants was recently described in which peptides are added to the CTA1 amino end (Fig. 3). The added peptides seem to reduce both enterotoxicity and ADP-ribosylating activity by sterical hindrance with the CTA1 active site [11]. In general, in these (like in all other detoxified) constructs, adjuvanticity decreased with decreasing enterotoxicity/ADP-ribosylation. However, a mutant (eCT6), with 10–20-fold lower enterotoxicity, displayed a level of adjuvanticity comparable to that of the wild type CT. Another mutant with a longer peptide linked to CTA1 (eCT23) and no detectable toxic activity, although being also much less potent in adjuvant activity than either CT or eCT6, was superior to CTB as both mucosal immunogen and as adjuvant for a co-administered antigen.

Yet another approach that is used to circumvent the harmful drawbacks of CT or LT adjuvants is to link the enzymatically active A subunit domain of the toxin to a cell-binding moiety other than the natural B subunit, such as the cell-binding domain of *Staphylococcus aureus* protein A (CTA1-DD) (Fig. 3) [12]. CTA1-DD, like most other toxin derivatives, functions when applied nasally but not when given orally. This limitation has recently been overcome by the incorporation of CTA1-DD fused to a short peptide into immune stimulating complexes (ISCOMS). Oral vaccination with the ISCOM-CTA1-DD complex induced systemic and mucosal responses with both Th1 and Th2 characteristics [13].

4. CT and CTB as adjuvants for dendritic cell vaccination

Dendritic cells (DC) are professional antigen-presenting cells, which act as sentinels throughout the body. Vaccination with autologous monocyte/bone-marrow-derived DC pulsed

with an appropriate antigen together with a suitable adjuvant ex vivo represents a promising approach to immunotherapeutic vaccination in e.g. patients with cancer or severe infections. The fact that the ex vivo-pulsed DC are autologous and can be extensively washed free from any unprocessed antigen and adjuvant before being reinfused to the patient makes it promising to also be able to use even such strong (but toxic) adjuvants as unmodified CT for stimulating the DC to become maximally immunogenic without risks of causing toxic side effects in vivo.

Conjugation of Ag to either CT or CTB greatly facilitated MHC class II-restricted antigen presentation by DC associated with both an upregulated secretion of IL-1 β by the pulsed DC and increased expression of CD80 and CD86 on the DC surface, effects that are linked to the adjuvant activity of CT. DC pulsed with CT- or CTB-conjugated antigen were found to be superior to DC pulsed with free antigen at inducing CD4 $^{+}$ T cell and B cell responses in vivo. However, whilst CTB-antigen conjugates almost exclusively stimulated immune responses with a Th2 type profile, conjugates based on CT were shown to drive the in vivo responses towards a mixed Th1 and Th2 phenotype (Fig. 4a) [10]. Vaccination with antigen-CT-pulsed DC, but not DC pulsed with antigen-CTB or antigen alone, also induced antigen-specific CD8 $^{+}$ T cells that produced IFN- γ and were cytotoxic for antigen (OVA)-expressing E.G7 tumour cells in vitro and were able to infiltrate and eliminate an already established OVA-expressing E.G7 tumour in vivo (Fig. 4b) [14,15]. We propose that the use of CT as a combined carrier-delivery system and adjuvant for DC vaccination represent a novel attractive strategy for therapeutic anti-tumour vaccination, at least for tumours that express sufficient amounts of MHC class I carrying tumour-specific peptide antigens.

5. CTB::CpG—a novel potent immuno-modulating adjuvant

Bacterial DNA contains unmethylated CpG motifs. This distinguishes bacterial DNA from vertebrate DNA, which contains a lower frequency of CpG motifs, and these are usually methylated [16,17]. Vertebrate immune systems appear to have evolved TLR9, that distinguishes bacterial DNA from self-DNA. Interactions between unmethylated CpG motifs in bacterial DNA or in synthetic oligodeoxynucleotide (ODN) and TLR9 rapidly activate DC, splenocytes, monocytes, and macrophages through the Toll/IL-1-receptor signaling pathway to produce Th1-polarizing cytokines such as IFN- γ , IL-1 β , tumor necrosis factor- α (TNF- α), and IL-12, to up-regulate co-stimulatory molecules on APCs, and to activate B cells for proliferation and IL-6 secretion. In vivo, systemic administration of CpG DNA was demonstrated to promote NK cell activity, to increase the total number of B-lymphocytes in the spleen, and to elevate plasma levels or tissue mRNA expression of IFN- γ , IL-12, and TNF- α . Altogether, CpG DNA induces a predominantly Th1 type immune activation.

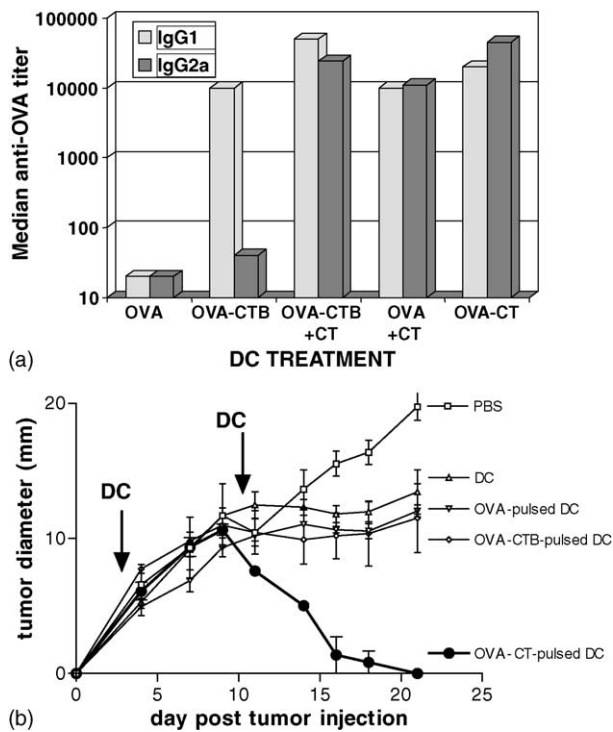


Fig. 4. Use of cholera toxin (CT) and cholera toxin B subunit (CTB) as adjuvants for dendritic cell vaccination: (a) Vaccination with bone-marrow derived dendritic cells (DC) pulsed ex vivo with a protein antigen (ovalbumin, OVA) in different formulations with or without CT or CTB in mice demonstrates a strong adjuvant action by coupling OVA to either CTB or CT on antibody responses and shows pronounced differential effects of CT and CTB on Th1 and Th2 responses: conjugation of antigen to CTB promotes a Th2 type of antibody response, whereas CT promotes a mixed Th1 and Th2 response (data from [10]), (b) DC vaccination with a tumor-specific antigen (OVA) coupled to CT can eliminate even an established tumor. Mice were subcutaneous inoculated with the OVA expressing E.G7 tumor cells, and then after 3 days and 10 days vaccinated with DC pulsed ex vivo with either OVA, OVA conjugated to CTB or OVA conjugated to CT. Vaccination with OVA-CT-pulsed DC efficiently eliminated the tumors (data from [14,15]).

Despite the documented effects of CpG DNA on systemic innate and adaptive immunity, little is known until recently about the role of CpG DNA in induction of innate immunity at mucosal surfaces. We have recently documented the impact of vaginal-mucosal administration of immunostimulatory CpG ODN on induction of innate immune responses in the murine female genital tract mucosa. Thus, a single mucosal-vaginal administration of immunostimulatory CpG ODN induces rapid production of Th1 associated cytokines IFN- γ , IL-12, and IL-18 in the female genital tract [18]. Further, such treatment stimulates rapid production of CC chemokines RANTES, MIP-1 α , and MIP-1 β in the murine female genital tract mucosa. The significance of the rapid CpG ODN-induced production of CC chemokines in the genital tract mucosa is highlighted by finding that infection of CD4 $^{+}$ T cells by HIV-1 is antagonized by RANTES, MIP-1 α , and MIP-1 β , the natural ligands of the HIV coreceptor CCR5 [19]. Strikingly, a single vaginal dose of CpG ODN, in the absence of any viral antigen, elicits protective immunity

against genital herpes infection and disease [18]. The protection led to the development of memory response conferring protection against reinfection. These data warrant further exploration for the use of immunostimulatory CpG ODN as a novel strategy to induce innate protective immunity against other sexually transmitted viral infections such as HIV infection.

It was also shown that immunostimulatory CpG DNA elicits innate immunity in the gastrointestinal tract mucosa. Thus, intragastric administration of mice with CpG ODN was found to elicit local production of CC chemokine RANTES, MIP-1 α and MIP-1 β and CXC chemokine IP-10 in the stomach and/or intestine. Importantly, intragastric administration of CpG ODN, in the absence of any exogenous bacterial antigen, suppressed bacterial colonization in the stomach of mice with an already established *Helicobacter pylori* infection [20].

In addition to the effect of CpG DNA on induction of innate immune response, several features of the immune stimulation induced by CpG DNA, including the generation of a Th1-like cytokine milieu, antiapoptotic activity on B cells and DCs, and upregulation of costimulatory molecule expression on antigen presenting cells suggest potential efficacy of CpG DNA as a vaccine adjuvant. The utility of CpG DNA as a potent Th1-like adjuvant for mucosal vaccination of primate and non-primate animals with various antigens, including hepatitis B surface antigen (intranasally and orally), whole killed influenza virus (intranasally or orally), MAP-M2, a synthetic measles virus peptide (intranasally), envelope glycoprotein B from HSV-1 (intranasally), tetanus toxoid (intranasally, intrarectally, orally), *Mycobacterium bovis* BCG (intranasally) have been demonstrated [21,22]. Importantly, some of these studies confirmed that CpG DNA is a stronger Th1-like adjuvant for inducing B cell and T cell responses than complete Freund's adjuvant, while the toxicity of CpG DNA was shown to be far less. It has also been reported that CpG covalently conjugated to protein antigen exhibits novel joint properties as promoters of antigen uptake and DC activation, thereby potentiating the ability of DCs to generate Th1-mediated immune response.

Most strikingly, it has recently been shown that the immunostimulatory effect of CpG DNA is significantly enhanced when chemically conjugated to CTB ([22], Harandi et al., to be published). When compared for their immunostimulatory effect on murine splenocytes, the CpG coupled to CTB induced much stronger chemokine responses than achieved with CpG alone, and when tested on human white blood cells even more pronounced differences were found. Thus, in vitro treatment of human peripheral blood mononuclear cells (PBMC) with an optimal human CpG ODN did not give rise to any appreciable levels of CC chemokine responses, CpG ODN coupled to CTB elicited strong CC chemokine responses by human PBMC. The corresponding optimal CTB-CpG conjugate when administered intravaginally, elicited stronger CC chemokine responses in the murine female genital tract mucosa compared with those of CpG ODN. The increased immunostimulatory property of CTB::CpG conju-

gate could be explained by the increased uptake of coupled CpG ODN by target cells and across the mucosal barrier and possibly also by CTB/GM1-directed intracellular transport of CpG into endoplasmic reticulum (Harandi et al., to be published).

6. Use of CTB for mucosal immunotherapy with proof-of-principle clinical study in patients with Behcet's disease

In many preclinical studies mucosal administration of antigens (Ag) linked to CTB has been shown to represent a very efficacious way of inducing peripheral tolerance (so-called oral tolerance) and thus suppressing the development of autoimmune or allergic reactions involving the coupled or bystander Ag [23–38]. The mechanisms for the strong potentiation of oral tolerance induction by using CTB-coupled Ag and not fully understood but appear to include the following CTB-mediated effects: (i) increased uptake of Ag across the mucosal barrier, (ii) increased Ag binding, uptake and presentation by DC and other APC, and (iii) providing selected DC (and other APC?) subsets with increased ability to induce different types of TGF- β expressing suppressor T cells including CD4⁺ CD25⁺ T regulatory T cells (Sun et al., to be published). This principle has now been tested in patients with Behcet's disease (BD). A BD-specific peptide (p336–351) is identified within the human 60 kDa heat shock protein (HSP60), and oral tolerization with the peptide linked to CTB prevented oral p336–351 induced uveitis in rats (Fig. 5) [29].

This strategy was therefore adopted in a phase I/II clinical trial by oral administration of p336–351::CTB, three times weekly for 12 weeks, followed by gradual withdrawal of all immunosuppressive drugs used to control the disease in eight patients with [39]. The patients were monitored by clinical and ophthalmological examination, as well as extensive immunological investigations. Oral administration of

p336–351::CTB had no adverse effect and withdrawal of the immunosuppressive drugs gave no relapse of uveitis in 5 of 8 patients or 5 of 6 selected patients who were free of disease activity prior to initiating the tolerization regimen. After tolerization was discontinued, 3 of 5 patients remained free of relapsing uveitis for 10–18 months after cessation of all treatment. Control of uveitis and extra-ocular manifestations of BD was associated with a lack of peptide-specific CD4⁺ T cell proliferation, a decrease in expression of Th1 type cells (CCR5, CXCR3), IFN- γ and TNF- α production, CCR7⁺ T cells and costimulatory molecules (CD40 and CD28), as compared with an increase in these parameters in patients in whom uveitis had relapsed.

The efficacy of oral peptide-CTB tolerization needs to be confirmed in a phase III trial, but this novel strategy in humans might be applicable generally to autoimmune diseases in which specific antigens have been identified.

References

- [1] Czerkinsky C, Holmgren J. The mucosal immune system and prospects for anti-infectious and anti-inflammatory vaccines. *Immunologist* 1995;3:97–103.
- [2] Czerkinsky C, Anjuere F, McGhee JR, et al. Mucosal immunity and tolerance: relevance to vaccine development. *Immuno Rev* 1999;170:197–222.
- [3] Wu HY, Weiner HL. Oral tolerance. *Immunol Res* 2003;28:265–84.
- [4] Cholera vaccines, WHO position paper. In: Weekly epidemiological record, vol. 76, Geneva, Switzerland: World Health Organization; 2001. p. 117–124.
- [5] Holmgren J, Bergquist C. Oral B subunit-killed whole cell cholera vaccine. In: Levine MM, Kaper JB, Rappuoli R, Liv MA, Good MF, editors. New generation vaccines. New York-Basel: Marcel Dekker; 2004. p. 499–510.
- [6] Holmgren J, Lycke N, Czerkinsky C. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector system. *Vaccine* 1993;11:1179–84.
- [7] Holmgren J, Harandi AM, Czerkinsky C. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Expert Rev Vaccines* 2003;2:205–17.
- [8] Pizza M, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan GKH, Rappuoli R, Del Giudice G. Mucosal vaccines: non-toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* 2001;19:2534–41.
- [9] Gagliardi MC, De Magistris MT. Maturation of human dendritic cells induced by the adjuvant toxin: role of cAMP on chemokine receptor expression. *Vaccine* 2003;21:856–61.
- [10] Eriksson K, Fredriksson M, Nordström I, Holmgren J. Cholera toxin and its B subunit promote dendritic cell vaccination with different influence on Th1/Th2 development. *Infect Immun* 2003;71:1740–7.
- [11] Sanchez J, Wallerstrom G, Fredriksson M, Angstrom J, Holmgren J. Detoxification of cholera toxin without removal of its immunoadjuvanticity by the addition of (STa-related) peptides to the catalytic subunit. A potential new strategy to generate immunostimulants for vaccination. *J Biol Chem* 2002;277:33369–77.
- [12] Agren LC, Ekman L, Lowenadler B, Nedrud JG. Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. *J Immunol* 1999;162:2432–40.
- [13] Mowat AM, Donachie AM, Jagewall S, et al. CTA1-DD-immune stimulating complexes: a novel, rationally designed combined mu-

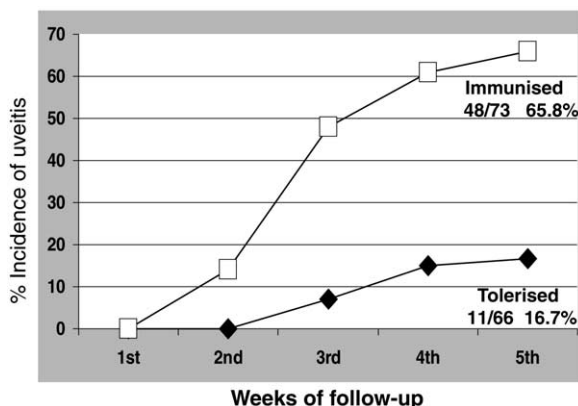


Fig. 5. Prevention of Behcet's disease. HSP60 p331–351 peptide induced uveitis in rats by oral tolerisation with CTB-p336–351 conjugate: The difference in incidence of uveitis in rats immunized but not tolerised vs. rats immunized and tolerised corresponds to a protective efficacy of 75%, $P < 0.0001$ (data from [29]).

- cosal vaccine adjuvant effective with nanogram doses of antigen. *J Immunol* 2001;167:3398–405.
- [14] Eriksson K, Sun JB, Nordström I, Fredriksson M, Lindblad M, Li BL, Holmgren J. Coupling of antigen to cholera toxin for dendritic cell vaccination promotes the induction of MHC class I-restricted cytotoxic T cells and the rejection of a cognate antigen-expressing model tumour. *Eur J Immunol* 2004;34:1272–81.
 - [15] Sun JB, Eriksson K, Li BL, Lindblad M, Azem J, Holmgren J. Vaccination with dendritic cells pulsed in vitro with tumor antigen conjugated to cholera toxin efficiently induces specific tumoricidal CD8⁺ cytotoxic lymphocytes dependant on cyclic AMP activation of dendritic cells. *Clin Immunol* 2004;112:35–44.
 - [16] Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002;20:709–60.
 - [17] Wagner H. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. *Curr Opin Immunol* 2002;5:62–9.
 - [18] Harandi AM, Eriksson K, Holmgren J. A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection. *J Virol* 2003;77:953–62.
 - [19] Cocchi F, DeVico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* 1995;270:1811–5.
 - [20] Raghavan R, Nyström J, Fredriksson M, Holmgren J, Harandi AM. Orally administered CpG oligodeoxynucleotide induces production of CXC and CC chemokines in the gastric mucosa and suppresses bacterial colonization in a mouse model of *Helicobacter pylori* infection. *Infect Immune* 2003;71:7014–22.
 - [21] Krieg A, Davis H. Enhancing vaccine with immune stimulatory CpG DNA. *Curr Opin Mol Ther* 2001;3:15–24.
 - [22] Harandi AM, Holmgren J. CpG DNA as a potent inducer of mucosal immunity: implications for immunoprophylaxis and immunotherapy of mucosal infections. *Curr Opin Investig Drugs* 2004;5:141–5.
 - [23] Sun J-B, Holmgren J, Czerkinsky C. Cholera toxin B subunit: an efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance. *Proc Natl Acad Sci* 1994;91:10795–9.
 - [24] Sun JB, Rask C, Olsson T, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proc Natl Acad Sci* 1996;93:7196–201.
 - [25] Sun J-B, Xiao B-G, Lindblad M, et al. Oral administration of cholera toxin B subunit conjugated to myelin basic protein protects against experimental autoimmune encephalomyelitis by inducing transforming growth factor- β -secreting cells and suppressing chemokine expression. *Int Immunol* 2000;12:1449–57.
 - [26] Bergerot I, Ploix C, Petersen J, et al. A cholera toxoid-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes. *Proc Natl Acad Sci* 1997;94:4610–4.
 - [27] Arakawa T, Yu J, Chong DK, Hough J, Engen PC, Langridge WH. A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nat Biotechnol* 1998;16:934–8.
 - [28] Tarkowski A, Sun J-B, Holmdahl R, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune arthritis by nasal administration of a type II collagen-cholera toxoid conjugate vaccine. *Arthritis Rheumatism* 1999;42:1628–34.
 - [29] Phipps PA, Stanford MR, Sun JB, et al. Prevention of mucosally induced uveitis with a HSP60-derived peptide linked to cholera toxin B subunit. *Eur J Immunol* 2003;33:224–32.
 - [30] Sun J-B, Li B-L, Czerkinsky C, Holmgren J. Enhanced immunological tolerance against allograft rejection by oral administration of allogeneic antigen linked to cholera toxin B subunit. *J Clin Immunol* 2000;97:130–9.
 - [31] Ma D, Mellon J, Niederkorn JY. Conditions affecting enhanced corneal allograft survival by oral immunization. *Invest Ophthalmol Vis Sci* 1998;39:1835–46.
 - [32] Tamura S, Hatori E, Tsuruhara T, Aizawa C, Kurata T. Suppression of delayed-type hypersensitivity and IgE antibody responses to ovalbumin by intranasal administration of *Escherichia coli* heat-labile enterotoxin B subunit-conjugated ovalbumin. *Vaccine* 1997;5:225–9.
 - [33] Rask C, Holmgren J, Fredriksson M, et al. Prolonged oral treatment with low doses of allergen conjugated to cholera toxin B subunit suppresses immunoglobulin E antibody responses in sensitized mice. *Clin Exp Allergy* 2000;30:1024–32.
 - [34] Li B-L, Sun J-B, Holmgren J. Adoptive transfer of mucosal T cells or dendritic cells from animals fed with cholera toxin B subunit alloantigen conjugate induces allogeneic T cell tolerance. *Adv Exp Med Biol* 2001;495:271–5.
 - [35] Boirivant M, Fuss U, Ferroni L, De Pascale M, Strober W. Oral administration of recombinant cholera toxin subunit B inhibits IL-12-mediated murine experimental (trinitrobenzene sulfonic acid) colitis. *J Immunol* 2001;166:3522–32.
 - [36] McSorley SJ, Rask C, Pichot R, Julia V, Czerkinsky C, Glaichenhaus N. Selective tolerization of Th1-like cells after nasal administration of a cholera toxoid-LACK conjugate. *Eur J Immunol* 1998;28:44–30.
 - [37] Sun J-B, Mielcarek N, Lakew M, et al. Intranasal administration of a *Schistosoma mansoni* glutathione S-transferase-cholera toxoid conjugate vaccine evokes antiparasitic and antipathological immunity in mice. *J Immunol* 1999;163:1045–52.
 - [38] Lebens M, Sun J-B, Mielcarek N, et al. A mucosally administered recombinant fusion protein vaccine against schistosomiasis protecting against immunopathology and infection. *Vaccine* 2001;3435:1–7.
 - [39] Stanford M, Whittall T, Bergmeier LA, Lindblad M, Lundin S, Shinnick T, Mizushima Y, Holmgren J, Lehner T. Oral tolerization with peptide 336–351 linked to cholera toxin B subunit preventing relapses of uveitis in Behcet's disease. *Clin Exp Immunol* 2004;137:201–8.