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Bacillus subtilis

A temperature resistant and needle free delivery system of immunogens

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Most pathogens enter the body through mucosal surfaces. Mucosal immunization, a non-invasive needle-free route, often stimulates a mucosal immune response that is both effective against mucosal and systemic pathogens. The development of mucosally administered heat-stable vaccines with long shelf life would therefore significantly enhance immunization programs in developing countries by avoiding the need for a cold chain or systemic injections. Currently, recombinant vaccine carriers are being used for antigen delivery. Engineering *Bacillus subtilis* for use as a non-invasive and heat stable antigen delivery system has proven successful. Bacterial spores protected by multiple layers of protein are known to be robust and resistant to desiccation. Stable constructs have been created by integration into the bacterial chromosome of immunogens. The spore coat has been used as a vehicle for heterologous antigen presentation and protective immunization. Sublingual (SL) and intranasal (IN) routes have recently received attention as delivery routes for therapeutic drugs and vaccines and recent attempts by several investigators, including our group, to develop vaccines that can be delivered intranasally and sublingually have met with a lot of success.

As discussed in this review, the use of *Bacillus subtilis* to express antigens that can be administered either intranasally or sublingually is providing new insights in the area of mucosal vaccines. In our work, we evaluated the efficacy of SL and IN immunizations with *B. subtilis* engineered to express tetanus toxin fragment C (TTFC) in mice and piglets. These bacteria engineered to express heterologous antigen either on the spore surface or within the vegetative cell have been used for oral, IN and SL delivery of antigens. A *Bacillus subtilis* spore coat protein, CotC was used as a fusion partner to express the tetanus fragment C. *B. subtilis* spores known to be highly stable and safe are also easy to purify making this spore-based display system a potentially powerful approach for surface expression of antigens. These advances will help to accelerate the development and testing of new mucosal vaccines against many human and animal diseases.

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Introduction

Although most vaccines are currently administered systemically, they are less effective against mucosal infections. Ideally, an efficient mucosal vaccine should provide protection not only at the mucosal delivery site but also systemically.[1] Mucosal vaccination can induce immune responses both at the mucosal surface usually by producing secretory IgA antibodies and at distant organs through systemic IgG production. The major antibody isotype found at mucosal sites and in external secretions is secretory IgA, predominantly in dimeric form, whereas the principle isotype found in the peripheral blood and in tissue spaces is IgG. A robust mucosal response is manifested by significantly higher fecal IgG and Secretory IgA (sIgA) responses and a mixed Th1/Th2 response as reflected by increased levels of interferon gamma and IL-2 cytokines and a balanced IgG1:IgG2a ratio. SIgA antibodies are considered major effectors in the adaptive immune defense of the mucosal system. More recently, the focus has shifted to mucosal vaccines capable of successfully generating both mucosal and systemic immune responses.[2] However, despite decades of extensive research, effective mucosal immunization remains elusive. Our understanding of mucosal immunity and development of mucosal vaccines has faced formidable challenges, with unpredictable results due to complex immune responses.[3] The mucosal immune system has also modified itself to thwart invasion and subsequent colonization by harmful microorganisms, to control transmission of pathogens between individuals and to prevent harmful immune reactions against food antigens and commensal bacteria.[4] The local microenvironment and the nature and route of antigen delivery are important determinants of the mucosal immune response.[2,5] Development of mucosal vaccines capable of effectively inducing both mucosal and systemic immune responses has been the focus of recent studies.[2,5] The IN route has been shown to induce strong systemic and secretory antibody responses and requires considerably smaller amount of antigen than would be required for oral administration.[6] However, some studies have related retrograde passage of inhaled antigens resulting in neurological side effects.[7-9] SL administration has been frequently used to deliver low-molecular-weight drugs, including small immunogenic peptides.[10-12] Unlike oral administration, SL administration avoids the enterohepatic circulation and degradation by gastric acids, rapidly delivering absorbed

antigen directly into the oral lymphoid tissue and into the blood stream simultaneously.

The use of live bacteria as vaccine delivery systems has provided one arm in the push to develop new and more effective vaccines. Bacterial endospores have shown potential as vehicles for delivery of heterologous antigens with previous studies demonstrating that orally and intranasally delivered *B.subtilis* spores expressing a *Clostridium tetani* antigen on the spore surface can protect mice against toxin challenge.[4,13] Other studies have shown that the *B. subtilis* spore can germinate in the murine gut and that this provides an additional route for antigen delivery.[14,15] The Gram-positive bacterium *B. subtilis* is currently used as a probiotic and a food additive and therefore has a proven safety record for humans.[16] In addition, it has the advantage of surviving in a metabolically dormant form indefinitely.[3] The *B.subtilis* spore is also considered to have adjuvant activity,[17] and is therefore useful for enhancing the delivery of heterologous antigens to the gastrointestinal tract.[18] Much knowledge is available on *B.subtilis* and it is also easy to genetically manipulate thereby facilitating construction of spores with ease.[19-22]

Various studies have confirmed the benefit of using the non-pathogenic, spore-forming bacterium *B. subtilis* as a non-invasive and highly thermostable, safe and low cost vaccine delivery system.[3,10,16] Studies by our group also confirmed the advantage of expressing microbial antigens in *B. subtilis* as compared with administration of purified antigens.[6] In addition to generating stronger and more protective immune responses, the bacteria make and deliver the antigen to the immunization site, minimizing the need for antigen production, purification, concentration, sterilization, packaging and the inclusion of adjuvants. In this Review, we provide an overview of the use of *B. subtilis* as an effective vaccine delivery system, administered either IN or SL as attractive alternatives to oral immunization. In IN and SL routes, the antigen is presented directly to the immune system without any need for germination of spores or vegetative cell replication. We then summarize our current research on the development of mucosal vaccines against tetanus using this *B. subtilis* as an immunogen delivery system.

Oral Immunization as a Vaccine Delivery Route

The oral route is considered superior for mucosal immunization involving protection of the gut and other mucosal surfaces because of the size of gastrointestinal surface compared with other organs, and safety, since the gut is able to handle and process toxic or substances more readily than other organs. Therefore, large amounts of antigen can be delivered by this route with minimal adverse effects. However, mucosal vaccines that are administered orally face hostile environment and a variety of host defenses. The mucosal secretions dilute them and they are broken down by gastric enzymes, proteases and nucleases. Also for oral immunization, relatively large doses of vaccine are required with no proper way of determining the actual quantity that crosses the mucosa or the precise site of uptake.[13] Oral immunization generates poor immune responses due to limited absorption and antigen degradation in the stomach.[23] As a result, vaccines that are capable of generating a robust immune response if injected parenterally are not as effective when given orally.[24] Consequently, only a few oral vaccines have been approved for human use. These include polio vaccine, *Salmonella typhi*, *Vibrio cholera* and rotavirus.[3,25-28,] Oral immunization can also lead to the development of tolerance where there is active suppression of systemic immunity due to the generation of various regulatory T cell responses. These T regulatory cells (TGF-β producing cells, IL-4 and IL-10 producing Th2) inhibit the generation of effector cells, and suppress disease by releasing antigen nonspecific cytokines, thereby resulting in systemic hypo-responsiveness.[29-31]

Our own results[6,32] suggested that oral administration is an ineffective way to deliver *B. subtilis* tetanus toxin fragment C (TTFC) vaccine strains, as we were unable to achieve reproducible protective immunity with this approach, even with 9–12 doses of spores or cells at $> 10^{10}$ per inoculation in mice or piglets. Oral immunizations failed to induce adequate antibody levels against tetanus or rotavirus and failed to protect animals against lethal tetanus or rotavirus challenges, respectively, indicating the strong correlation between antibody production and protection (data not published).

Intranasal Immunization

IN administration is an attractive alternative to oral immunization, specifically for surface-expressed antigens. Low doses of antigen are presented directly to the nasopharyngeal immune system and induce stronger systemic immune responses than the oral route.[33,34] In various studies in mice, monkeys and humans, nasal administrations of vaccines induced specific mucosal IgA antibody responses and cytotoxic T lymphocytes in the respiratory and genital tracts, the gastrointestinal tracts and salivary glands.[3,13,35-37] Intranasal immunization studies in humans and mice produced greater systemic antibody responses than other mucosal immunization routes,[3,6,41] probably because antigens or antigen-presenting cells were readily trafficked to draining lymph nodes from this site. In our studies, IN immunization with even relatively low doses of TTFC expressing recombinant *B. subtilis* spores or vegetative cells induced robust and consistent systemic immunity, with high titers of serum antibody that were highly protective against lethal challenge with tetanus toxin. In their lyophilized forms, the vaccine strains maintained full protective immunogenicity for at least 12 mo at 45°C.[6,38]

However, a current concern for live nasal vaccines is the possibility of retrograde migration to the brain through olfactory nerves, as has been found with live attenuated adenovirus.[8] Murine studies have demonstrated that cholera toxin (CT), when administered IN as a mucosal adjuvant can redirect co-administered vaccine antigen into the CNS: olfactory nerves/epithelium and brain.[23] The speculation is that use of adjuvants could increase the risk of sensitization via stimulation of Th2-biased responses leading to organisms crossing the blood brain barrier.[14] Facial nerve fibers might also absorb the adjuvant, leading to retrograde transport and neuronal damage. Song et al.[15] demonstrated that intranasal administration of live and inactivated influenza virus resulted in the accumulation of antigens in the

olfactory bulb and brain within 24 h. Such safety concerns appear to limit the usefulness of the IN route in humans. Consequently, only one vaccine has been approved for IN administration, the live attenuated cold adapted trivalent IN influenza virus vaccine in children. To date no side effects have been reported.[28]

Sublingual Immunization

SL immunization against infectious agents or bacterial toxins is not a common route for antigen delivery. However, it is currently receiving attention as a novel delivery site for therapeutic drugs and vaccines. As opposed to oral administration, SL administration of proteins and peptides is a convenient way to deliver them in minute quantities to the bloodstream, in addition to the local oral lymphatics. The enterohepatic circulation is avoided and effects of acid and partial first pass hepatic metabolism are bypassed.[39]

SL immunization has been used for many years as a non-invasive and effective immunotherapy for the treatment of allergies. Antigens are absorbed quickly; enter the bloodstream, bypassing the liver and intestine, thereby eliciting allergen specific tolerance.[40] Histologically, the SL epithelium has a dense network of dendritic cells: antigen presenting cells, which have been shown to rapidly increase after topical application of antigen under the tongue.

Previous studies have demonstrated that SL administration of ovalbumin delivered with cholera toxin as adjuvant induces a broad range of immune responses in mucosal and extra mucosal tissues, including secretory and systemic antibody responses and cytotoxic T lymphocytes.[4] In another study, SL administration of live or inactivated influenza virus protected mice against influenza virus. Protection was associated with mucosal and systemic immune responses, including antibody production and cytotoxic T lymphocyte expansion.[40,41] In yet another study, SL immunization was found to induce vaccine-specific antibody and T-cell responses in the genital tract and, after SL immunization with human papillomavirus (HPV)-like particles, protection against genital HPV infection, indicating the potential of SL immunization to stimulate immune responses also in non-respiratory mucosal tissues.[42] Notably, in contrast to the IN route, either inactivated or live influenza virus did not migrate to or replicate in the CNS, after SL administration.[41] In our studies, we were able to demonstrate that sublingual administration of tetanus vaccine was effective in inducing a robust protective immune response against tetanus toxin challenge.[6,32,38] We also demonstrated that, at least in the case of tetanus, for both SL and IN immunizations, the inclusion of the adjuvant mLT was detrimental.[6,32] SL immunization was used as an effective route for delivery of antigens. In addition to the non-invasive aspect of the delivery method, SL is superior to oral administration in infants and children in terms of simplicity, safety, volume required and consistency of outcome, avoiding the hazards of digestion and concurrent diarrheal illness, which often reduces vaccine efficacy. A major advantage of Sublingual immunization is that it induced secretory IgA antibodies that were detected in the saliva, vaginal wash and fecal content. The level of IgG in the gut was also high although it was not clear whether this was locally generated or a leakage of circulating antibody. As previously mentioned, IgA antibodies are considered major effectors in generating a robust mucosal response. Although the protective immunization with TTFC expressed in *B. subtilis* was against tetanus, a systemic intoxication, the levels of mucosal antibody responses at different sites were impressive, indicating that SL and IN routes of immunizations were equally effective against systemic and mucosal agents.

Using Recombinant *B. subtilis* as Vaccine Delivery Vehicle

Several recent studies have shown that *Bacillus subtilis* spores and cells engineered to express vaccine antigens can be used effectively to generate systemic and mucosal antibodies.[43-47] We have demonstrated that *B. subtilis* vegetative cells and spores engineered to express tetanus toxin fragment C (TetC) can induce protective immunity in mice and piglets, and when stored as lyophilized powders, have long-term stability during storage at elevated temperatures.[6,32,38] In these experiments, these vaccines are effective when administered either intranasally or sublingually. The rationale behind the development of improved vaccination strategies is to provide better levels of local immunity against pathogens which enter the body primarily through the mucosal surfaces, offer needle-less routes of administration with improved safety and minimal adverse side effects, and provide economical vaccines for developing countries where suboptimal storage and transportation facilities reduce the effectiveness of immunization programs. Different carrier systems have been developed to improve mucosal immune responses, nonliving systems include liposomes, micro particles, immunity-stimulating complexes, and formulations based on cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxins (LT).[48] Live carrier systems include plants, bacteria, and viruses. Although bacterial systems for heterologous antigen presentation have been considered, safety concerns remain due to use of live attenuated pathogens such as salmonella and mycobacteria.[48] With the increased knowledge of mucosal immunity and the availability of genetic tools for heterologous gene expression, the concept of live vaccine vehicles has gained renewed interest.[49] Genetically engineered bacterial spores and vegetative cells offer promise as both mucosal as well as a heat-stable vaccine delivery system.[49] Bacterial spores have been shown to be effective as recombinant vaccine vehicles, where an antigen is expressed on the spore surface or within the germinating vegetative cell. Spores offer the added advantage of their unique heat-stability and therefore are convenient for use in developing countries. Spores are robust and dormant life forms with formidable resistance properties and unlike many second generation vaccine systems currently under development, both spores and vegetative cells offer the flexibility for genetic manipulation.[50]

Spores of different *Bacillus* species are used as probiotics in animals and humans,[46,47,51] and in some regions of Asia and Africa there is a widespread consumption of spore-based foods.[52] *Bacillus* spores, including *B. subtilis*, are being widely used as dietary

supplements with a number of species registered for human use, including *B. subtilis*, *B. cereus*, *B. clausii*[53] and most recently, *B. coagulans*. Probiotics are commonly fed to piglets to stabilize the gut micro flora as a preventive measure during the critical period of weaning.[54] They maintain or enhance the indigenous defense mechanisms in the animal without disturbing normal physiological or biochemical functions.[55,56]

B. subtilis engineered to express heterologous antigens on the surface of the spore or within the vegetative cell can be used for oral or nasal delivery of antigens and confer protective immunity.[6,55-59] Studies performed demonstrated that orally and intranasally delivered *B. subtilis* spores expressing a *Clostridium tetani* antigen on the spore surface can protect mice against toxin challenge.[6,32,38] Other studies have shown that the *B. subtilis* spore can germinate in the murine gut and that this provides an additional route for antigen delivery.[60,61]

B. *subtilis* produces an endospore as part of its developmental life cycle when starved of nutrients and the mature spore can survive for long periods of time in a dormant form.[62] It can also survive temperature extremes, and exposure to solvents. These unique attributes make the spore an attractive vehicle for delivery of heterologous antigens or any bioactive molecule to extreme environments such as the gastrointestinal tract. In addition, *B. subtilis* has been recently shown to be important for the development of the gut-associated lymphoid tissue.[63]

Physical features of spores, together with biological properties, such as safety record in humans and animals[52,53] and their ability to interact with antigen presenting cells and to stimulate cytokine release,[21,64,65] make Bacillus spores extremely interesting candidates as vaccine vectors.[21,65] Furthermore, large-scale production is inexpensive, and genetic tools as well as complete genomic data are available.[64] In our studies, a genetic system for the construction of recombinant *B. subtilis* spores and vegetative cells expressing heterologous antigens on the spore surface and in the vegetative cell was developed.[6,64] The spore coat protein CotC was used as fusion partner for the surface display of tetanus toxin fragment C (TTFC), a well-characterized and highly immunogenic model antigen.[66,67] *B. subtilis* strains were also developed to express TTFC in the cell cytoplasm. These were constructed to either have one copy or three copies of a Pspac1/2-TTFC transcriptional fusion at one or three different chromosomal loci. The immunogenicity of TTFC expressed on the spore surface or within the vegetative cell cytoplasm was demonstrated in mice and piglets immunized by the SL and IN routes.[6,32,38]

Adjuvant Activity of *B. Subtilis*

Another objective in the development of better vaccines is to identify new adjuvants that will enhance the immunogenic activity of weak antigens. Vaccines are effective against preventing infectious diseases. However, vaccines induce only suboptimal immunity, and need multiple boosts to generate a robust antibody response (e.g., anthrax, diphtheria, pertussis and tetanus).[6,59,68] This suggests a critical need for more efficacious adjuvants. Bacterial toxins are considered the most powerful adjuvants when mucosally delivered in experimental animal models.[69] They are the gold standard in vitro and preclinical models and for evaluating and analyzing mucosal routes of vaccine delivery. Due to their toxicity in humans, nontoxic derivatives of cholera toxin and labile toxin have been identified.[69]

Previous studies have shown that Bacillus spores possess adjuvant properties,[56,70] and this is brought about by binding of the antigen to the spore surface.[56] In one study, Barnes et al.,[56] used probiotic *B. subtilis* spores, known to be safe and fully tolerated by ingestion in man, and explored their ability to influence the magnitude and diversity of immune responses induced against two model antigens, tetanus toxoid fragment C (TT) and ovalbumin (OVA) in mice. The results showed that *B. subtilis* spores not only increased antibody and T cell responses to a co-administered soluble antigen, but also broadened them, to include both antigen-specific $CD4^+$ and $CD8^+$ T cell responses, as well as complement and non-complement fixing antibody isotopes. Furthermore, following IN immunization, spores augmented specific IgA to co-administered antigen both in the local respiratory and distal vaginal mucosa, as well as increased antigen-specific IgG antibody in draining LN and blood. In the same study, while immunization with tetanus toxoid (TT) alone induced an IgG1-dominated response, which was maintained after three immunizations, co-administration of spores with TT resulted in significant enhancement of both TT-specific IgG1 and IgG2a antibody titers.[56] A balanced IgG1:IgG2aration is significant in generating a mixed Th1/Th2 response. Therefore, *B. subtilis* spores enhance antibody responses to co inoculated antigens with a far greater efficiency than they do to self. The data suggests that *B. subtilis* spores enhance both humoral and cellular antigen-specific responses, and induce a balanced Th1/Th2 response making them useful as adjuvants.

Bacillus Subtilis as a Temperature Resistant and Needle Free Delivery System of Immunogens

Foreign antigens have been expressed in *B. subtilis* on the spore surface and within the vegetative cells. Genetic tools for the efficient expression and secretion of recombinant proteins in *B. subtilis* have been rapidly developed and successfully used.[6,71,72] Detailed morphological and genetic studies have shown that the *B. subtilis* spore is surrounded by a multilayered coat,[62] whose proteinacious nature suggests the possibility of using its structural components as fusion partners for the expression of heterologous proteins on the spore surface. Both vegetative cells and spores have been used as delivery vectors but the primary model used to date has been the spore form of *B. subtilis* displaying tetanus toxin antigen. Acheson et al. first considered the option of engineering *B. subtilis* for use as a non-invasive, heat and environmentally-resistant antigen delivery system.[73] In their report of a *B. subtilis* spore-based vaccine they found that oral administration of spores expressing the invasin antigen of *Yersinia pseudotuberculosis* in the replicating vegetative stage of growth induced a systemic antibody response in mice. Other groups have successively pursued this idea with encouraging results.[37,41] Mauriello et al.[75] went on to use a spore coat protein to express tetanus toxin fragment C (TTFC). They reported that recombinant spores expressing TTFC on their

surface given either orally or intranasally induced antigen-specific immune responses independent of their ability to germinate in the gastrointestinal tract. The spore surface as a fusion product with spore coat proteins has been shown to elicit protective immune responses.[5]

Studies performed by our group in mice and piglets provided evidence that immunization with spores or vegetative cell preps of *B. subtilis* expressing tetanus toxin stimulated both a systemic and mucosal response.[3,32,38] In our studies, we were able to show that in both mice and piglets, SL and IN administration of a recombinant *B. subtilis* expressing the tetanus toxin fragment C induced vigorous systemic and mucosal immune responses, including a local mucosal response in the SL and IN tissues as well as disseminated mucosal antibody responses in distant organs such as the lungs, intestines and reproductive organs. Animals immunized 3–4 times with a *B. subtilis* expressing TTFC via the SL and IN routes generated robust IgG antibody titers and significantly higher IgA titers compared with animals that received the standard DTaP vaccine intramuscularly. The induction of mucosal antigen specific IgA production was detected in the saliva, vaginal washes and in the feces.

SL and IN administration of TTFC expressed in *B. subtilis* in the absence of an adjuvant was capable of inducing persistent systemic and mucosal immune responses up to 10 weeks post immunization. Comparable levels of anti-TT IgG titers were detected in animals that received mutant labile toxin from *E. coli* (LT) as adjuvant and those that did not, indicating that an adjuvant is not required. Other studies have shown that in IN immunization, *B. subtilis* spores augmented specific IgA to co-administered antigen both in the local respiratory and distal vaginal mucosa, as well as increased antigen-specific IgG antibody in draining LN and blood.

Ideally, an efficient mucosal vaccine should induce antibody mediated protection, not only at the mucosal delivery site but also throughout the body including systemic compartments as well as distal mucosal tissues. In our studies, anti-TT IgA titers were detectable and higher in the fecal, saliva and vaginal washes of animals immunized via the SL and IN routes with *B. subtilis* expressing tetanus toxin antigen compared with animals that received the commercial DTaP vaccine intramuscularly. We also determined that significantly higher levels of cytokines IFN-γ and IL-2 were produced in the SL and IN groups compared with the DTaP group thereby indicating a mixed Th1/Th2 response in the mucosally immunized animals. More studies proved that IN or SL immunizations of mice with a *B. subtilis* recombinant expressing TTFC (either spore based or vegetative cell based expression) provided as much protection as the standard injectable DTaP against a systemic lethal toxin challenge.[32,38] In piglets, studies done provided additional evidence that SL and IN immunization induced local and systemic immune responses. A passive neutralization assay in mice injected with sera from SL immunized pigs documented that the antibodies generated in pigs were toxin neutralizing and protective.[6,32,38] The results in pigs provided encouragement that humans will respond as well, as the immune system in pigs is much more like the human immune system than is murine immunity.

In other studies, we were able to demonstrate that other antigens such as the rota virus VP6 and the botulinum BoNT antigen can be transformed into *E. Coli* and that the VP6 antigen could be successfully displayed on the spore surface of *B. subtilis* (data not published). Using a fusion partner CotC, the shuttle vector pMK3 was able to successfully express and expose on the spore surface VP6, a protein of rotavirus. CotC, an alkali soluble component of the *B. subtilis* spore coat,[71] is considered as a carrier candidate because of its abundant nature. Together with CotG and CotD, CotC represents about 50% of the total solubilized coat proteins.[71] Such relatively high amounts could allow the assembly of a significant number of CotC-based chimeras on the coat, thus ensuring an efficient heterologous display. Rotavirus VP6 antigen was expressed as a CotC-VP6 fusion protein in the *B. subtilis* spores. Other groups have been able to express BoNT/A in *E. Coli*.[72] Byrne et al. developed a method for purification of the Hc polypeptide expressed from the synthetic gene in yeast *Pichia pastoris*.[76] Sonenshein et al. have been able to express the BoNT/A gene in the chromosome of *B. subtilis* to make a single copy or double copy construct.[6] The BoNT serotypes and the serotype for tetanus toxin are structurally and functionally related, sharing both amino acid sequence homology, as well as structural homology.[77-79] So much of the work on recombinant botulism vaccines is based on the approach used for the recombinant tetanus vaccine. This work confirms that spores can be engineered and developed as vaccine carriers.

Although tetanus vaccines protect against infection by inducing serum IgG antibodies, SL and IN administered antigen induced both mucosal and systemic protection, probably as a result of the enhanced SIgA responses at mucosal sites. SL and IN immunization with recombinant *B. subtilis* expressing TTFC was as effective for priming T cells in regional lymph nodes and spleen and promoted regional and systemic mixed Th1 and Th2 responses. T cells from spleen and submandibular lymph nodes of piglets given antigen via the SL route showed significantly higher proliferative responses when compared with piglets immunized with a placebo. Analysis of cytokines showed that increased levels of IFN-γ, IL-2, IL-4 and IL-10 were detected in the sera and in specific tissues isolated from both mice and piglets given antigen via SL routes compared with IN, oral routes and to animals that received the vaccine DTaP IM. IgG subclass responses in mice confirmed the cytokine profile showing that recombinant TTFC expressing *B. subtilis* elicited both IgG1 and IgG2a anti-TT antibody responses.

Efficient induction of T cell responses was further supported by the presence of large numbers of MHC class II+ staining cells in the salivary glands, submandibular lymph nodes draining the immunization site, lungs, spleen and intestines; the last three organs were not the site of induction. The presence of high numbers of MHC class II+ staining cells was an indication of presence of dendritic cells, which are able to capture, process and present antigen to either or both $CD4^+$ or $CD8^+$ T cells. SL immunization was also able to induce CD3 cell responses in the salivary glands within 24 h after re-stimulation with TT antigen.

While sublingual immunization with rTTFC alone induced an IgG1-dominated response, which was maintained after three

immunizations, immunization with TTFC expressing *B. subtilis* resulted in significant enhancement of both TT-specific IgG1 and IgG2a antibody titers. The increase in TT-specific IgG2a relative to IgG1 observed following mucosal immunization with TTFC expressing *B. subtilis* resulted in a significant shift from a Th2-dominated IgG1 response, toward a balanced distribution of IgG1 and IgG2a isotypes, hence a mixed Th1/Th2 response. This was not achieved with DTaP or with rTTFC.

B. subtilis seemed to play an important role in the induction of a balanced Th1/Th2 response. The ratio of isotope IgG1 to IgG2a antibodies in mice that received naked recombinant TTFC antigen given via SL route was significantly higher compared with *B. subtilis* TTFC- SL and to *B. subtilis* TTFC-IN.[6,32,38] Although it was still much lower that the DTaP administered IM, it is evident that *B. subtilis* was essential. While promoting heightened antibody and T cell responses is an established requisite of an adjuvant, the induction of a balanced Th1 and Th2 responses is equally important.[38] In our work, we deduced from antibody isotype profiles a balanced IgG isotype, redolent of a mixed Th1/Th2 profile. In this way, both Th1 and Th2 responses were generated to the target antigens, which were neither Th2 dominated with a loss of cellular immunity or Th1 dominated with the risk of autoimmunity. One explanation for this observation is that the spores efficiently introduce antigen directly into the MHC class I (as well as class II) presentation pathway. This will lead to the induction of a balanced Th1/Th2 response. *B. subtilis* also had an adjuvant function. It is well known that the role of adjuvant is particularly essential in inducing a good immune response to the co-administered antigen. In our studies, we found that higher anti-TT antibody titers were observed in mice that did not have mLT as adjuvant compared with those that received adjuvant. Such immunogenic properties correlates with *B. subtilis* ability to bind to several types of APC's including B cells, dendritic cells and macrophages and induce both B cell and T cell activity.

One of the greater worries about using *B. subtilis* is the fear that vegetative cells or spores may accidentally enter the lung tissue and replicate within them, or segments may migrate to the brain tissue and cause neurological damage. In our experiments, mice were immunized via the SL and IN route and terminated at 2, 6 and 24 h time points post inoculation, to determine if there were spores germinating in the lung tissue and whether there was any infiltration of inflammatory cells either in the lungs or in the brain. Culture of the lung and brain tissue did not show any spores or vegetative cells present. Gross examination showed that the lungs and brain looked as normal as the control animals. Histological examination of the same tissue did not reveal any signs of inflammation in either brain or lung tissue or the presence of spores (data not published).

We were therefore able to demonstrate that there was no germination of vegetative cells within the lung of SL immunized animals and both lung and brain tissue did not show any inflammatory reaction. In this regard, Song et al.[15] have shown that after SL administration, live or inactivated influenza virus does not migrate into the central nervous system.

Conclusion

The use of spores as a vaccine carrier provides a major step forward in the search for new and effective vaccine delivery system that is temperature resistant and non-invasive. *B. subtilis* can easily be genetically manipulated, and allows for the expression and display of different immunogens such as a fragment of tetanus toxin or rotavirus on the surface of the spore or in the vegetative cell.[4] These *B. subtilis* based vaccines can be stored for long periods of time and are readily produced at low cost in large fermenters thereby offering an attractive second-generation vaccine vehicle. The bacteria form heat-resistant spores that can be stored without loss of viability at ambient temperatures eliminating the need for cold storage. The stability of the heterologous proteins exposed has been investigated and was found to be stable and still efficacious even at 17 mo.[32,38]

This display system has been used to express tetanus toxin fragment C (TTFC), the highly immunogenic C fragment of tetanus toxin which is used as a model immunogen. The TTFC could be expressed both within the vegetative cell and on the spore coat of *B. subtilis*. In our studies, we were also able to use it to express the rotavirus VP6 and the Botulinum antigen BoNT. These results indicate that heterologous proteins can be stably exposed on the surface of *B. subtilis* and confirm it as a promising delivery system for vaccines.

Other studies have demonstrated, at least in the case of tetanus, for both SL and IN immunizations, that adjuvant is not required with *B. subtilis*. Adjuvant activity was evident when TTFC expressed in *B. subtilis* administered SL was compared with naked recombinant TTFC. Other studies[17] however did show that SL immunization with tetanus toxoid required the inclusion of the adjuvant LT for antibody production, although in the published experiments mice were not challenged to establish protection.

The lyophilized TTFC-expressing *B. subtilis* vegetative cell vaccine has been seen to be stable for at least 17 mo of incubation at 45°C[6,32,38] retaining full immunogenicity by either IN or SL immunization and giving full protection against toxin challenge.

The non-pathogenic, spore-forming bacterium *B. subtilis* has been effectively used as a non-invasive and highly thermo stable, safe and low cost vaccine delivery system.[9-11] Many studies also confirm the advantage of expressing microbial antigens in *B. subtilis* as compared with administration of purified antigens. In addition to generating stronger and more protective immune responses, the bacteria make and deliver the antigen to the immunization site, minimizing the need for antigen purification, concentration, sterilization, packaging, and the inclusion of adjuvants. The fact that antigens can be administered sublingually and intranasally, offers a significant advantage compared with the current conventional methods of parenteral and oral vaccination against systemic and mucosal diseases, respectively.

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References

1. Negri DR, Riccomi A, Pinto D, Vendetti S, Rossi A, Cicconi R, et al. Persistence of mucosal and systemic immune responses following sublingual immunization. Vaccine 2010; 28:4175-80; PMID:20412876; http://dx.doi.org/10.1016/j.vaccine.2010.04.013
2. Yuki Y, Kiyono H. New generation of mucosal adjuvants for the induction of protective immunity.
3. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 2006; 6:148-58; PMID:16491139; http://dx.doi.org/10.1038/nri1777
4. Cuburu N, Kweon MN, Song JH, Hervouet C, Luci C, Sun JB, et al. Sublingual immunization induces broad-based systemic and mucosal immune responses in mice. Vaccine 2007; 25:8598-610; PMID:17996991; http://dx.doi.org/10.1016/j.vaccine.2007.09.073
5. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005; 11(Suppl):S45-53; PMID: 15812489; http://dx.doi.org/10.1038/nm1213
6. Lee S, Belitsky BR, Brown DW, Brinker JP, Kerstein KO, Herrmann JE, et al. Efficacy, heat stability and safety of intranasally administered Bacillus subtilis spore or vegetative cell vaccines expressing tetanus toxin fragment C. Vaccine 2010; 28:6658-65; PMID: 20709005; http://dx.doi.org/10.1016/j.vaccine.2010.08.016
7. Armstrong ME, Lavelle EC, Loscher CE, Lynch MA, Mills KH. Proinflammatory responses in the murine brain after intranasal delivery of cholera toxin: implications for the use of AB toxins as adjuvants in intranasal vaccines. J Infect Dis 2005; 192:1628-33; PMID: 16206078; http://dx.doi.org/10.1086/491739
8. Lemiale F, Kong WP, Akyürek LM, Ling X, Huang Y, Chakrabarti BK, et al. Enhanced mucosal immunoglobulin A response of intranasal adenoviral vector human immunodeficiency virus vaccine and localization in the central nervous system. J Virol 2003; 77:10078-87; PMID:12941918; http://dx.doi.org/10.1128/JVI.77.18.10078-10087.2003
9. Fujihashi K, Koga T, van Ginkel FW, Hagiwara Y, McGhee JR. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. Vaccine 2002; 20:2431-8; PMID:12057597; http://dx.doi.org/10.1016/S0264-410X(02)00155-X
10. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005; 11(Suppl):S45-53; PMID: 15812489; http://dx.doi.org/10.1038/nm1213
11. BenMohamed L, Belkaid Y, Loing E, Brahimi K, Gras-Masse H, Druilhe P. Systemic immune responses induced by mucosal administration of lipopeptides without adjuvant. Eur J Immunol 2002; 32:2274-81; PMID: 12209640; http://dx.doi.org/10.1002/1521-4141(200208)32:8<2274::AID-IMMU2274>3.0.CO;2-C
12. Zhang H, Zhang J, Streisand JB. Oral mucosal drug delivery: clinical pharmacokinetics and therapeutic applications. Clin Pharmacokinet 2002; 41:661-80; PMID: 12126458; http://dx.doi.org/10.2165/00003088-200241090-00003
13. Kozlowski PA, Williams SB, Lynch RM, Flanigan TP, Patterson RR, Cu-Uvin S, et al. Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. J Immunol 2002; 169:566-74; PMID:12077289
14. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J Immunol 2000; 165:4778-82; PMID:11045998
15. Song JH, Nguyen HH, Cuburu N, Horimoto T, Ko SY, Park SH, et al. Sublingual vaccination with influenza virus protects mice against lethal viral infection. Proc Natl Acad Sci U S A 2008; 105:1644-9; PMID:18227512; http://dx.doi.org/10.1073/pnas.0708684105
16. Kozlowski PA, Cu-Uvin S, Neutra MR, Flanigan TP. Comparison of the oral, rectal, and vaginal immunization routes for induction of antibodies in rectal and genital tract secretions of women. Infect Immun 1997; 65:1387-94; PMID:9119478
17. Macdonald TT, Monteleone G. Immunity, inflammation, and allergy in the gut. Science 2005; 307:1920-5; PMID:15790845; http://dx.doi.org/10.1126/science.1106442
18. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 2004; 118:229-41; PMID:15260992; http://dx.doi.org/10.1016/j.cell.2004.07.002
19. Hong HA, Duc H, Cutting SM. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 2005; 29:813-35; PMID:16102604; http://dx.doi.org/10.1016/j.femsre.2004.12.001
20. Green DH, Wakeley PR, Page A, Barnes A, Baccigalupi L, Ricca E, et al. Characterization of two Bacillus probiotics. Appl Environ Microbiol 1999; 65:4288-91; PMID:10473456
21. Oggioni MR, Ciabattini A, Cuppone AM, Pozzi G. Bacillus spores for vaccine delivery. Vaccine 2003; 21 (Suppl 2):S96-101; PMID:12763690; http://dx.doi.org/10.1016/S0264-410X(03)00207-X
22. Negri DR, Riccomi A, Pinto D, Vendetti S, Rossi A, Cicconi R, et al. Persistence of mucosal and systemic immune responses following sublingual immunization. Vaccine 2010; 28:4175-80; PMID:20412876; http://dx.doi.org/10.1016/j.vaccine.2010.04.013
23. Detmer A, Glenting J. Live bacterial vaccines–a review and identification of potential hazards. Microb Cell Fact 2006; 5:23; PMID:16796731; http://dx.doi.org/10.1186/1475-2859-5-23
24. Izadpanah A, Dwinell MB, Eckmann L, Varki NM, Kagnoff MF. Regulated MIP-3alpha/CCL20 production by human intestinal epithelium: mechanism for modulating mucosal immunity. Am J Physiol Gastrointest Liver Physiol 2001; 280:G710-9; PMID: 11254498
25. Modlin JF. Poliomyelitis in the United States: the final chapter? JAMA 2004; 292:1749-51; PMID:15479943; http://dx.doi.org/10.1001/jama.292.14.1749
26. Levine MM, Kaper JB. Live oral vaccines against cholera: an update. Vaccine 1993; 11:207-12; PMID: 8438619; http://dx.doi.org/10.1016/0264-410X(93)90019-T
27. Rennels MB, Glass RI, Dennehy PH, Bernstein DI, Pichichero ME, Zito ET, et al. United States Rotavirus Vaccine Efficacy Group. Safety and efficacy of high-dose rhesus-human reassortant rotavirus vaccines–report of the National Multicenter Trial. Pediatrics 1996; 97:7-13; PMID:8545227
28. Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, Piedra P, et al. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. N Engl J Med 1998; 338:1405-12; PMID:9580647; http://dx.doi.org/10.1056/NEJM199805143382002
29. Iwasaki A, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J Exp Med 1999; 190:229-39; PMID:10432286; http://dx.doi.org/10.1084/jem.190.2.229
30. Wu HY, Weiner HL. Oral tolerance. Immunol Res 2003; 28:265-84; PMID:14713719; http://dx.doi.org/10.1385/IR:28:3:265
31. Miller A, Lider O, Roberts AB, Sporn MB, Weiner HL. Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. Proc Natl Acad Sci U S A 1992; 89:421-5; PMID: 1370356; http://dx.doi.org/10.1073/pnas.89.1.421
32. Amuguni H, Lee S, Kerstein KO, Brown DW, Belitsky BR. Herrmann JE, Keusch GT, Sonenshein AL, Tzipori S. Sublingual immunization with an engineered Bacillus subtilis strain expressing tetanus toxin fragment C induces systemic and mucosal immune responses in piglets. [Epub ahead of print]. Microbes Infect 2011; PMID:22198093
33. Bergquist C, Lagergård T, Holmgren J. Anticarrier immunity suppresses the antibody response to polysaccharide antigens after intranasal immunization with the polysaccharide-protein conjugate. Infect Immun 1997; 65:1579-83; PMID:9125533
34. Johansson EL, Rask C, Fredriksson M, Eriksson K, Czerkinsky C, Holmgren J. Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates. Infect Immun 1998; 66:514-20; PMID: 9453604
35. Staats HF, Montgomery SP, Palker TJ. Intranasal immunization is superior to vaginal, gastric, or rectal immunization for the induction of systemic and mucosal anti-HIV antibody responses. AIDS Res Hum Retroviruses 1997; 13:945-52; PMID:9223410; http://dx.doi.org/10.1089/aid.1997.13.945
36. Imaoka K, Miller CJ, Kubota M, McChesney MB, Lohman B, Yamamoto M, et al. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. J Immunol 1998; 161:5952-8; PMID:9834076
37. Rudin A, Riise GC, Holmgren J. Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. Infect Immun 1999; 67:2884-90; PMID:10338495
38. Amuguni JH, Lee S, Kerstein KO, Brown DW, Belitsky BR, Herrmann JE, et al. Sublingually administered Bacillus subtilis cells expressing tetanus toxin C fragment induce protective systemic and mucosal antibodies against tetanus toxin in mice. Vaccine 2011; 29:4778-84; PMID:21565244; http://dx.doi.org/10.1016/j.vaccine.2011.04.083
39. Cuburu N, Kweon MN, Hervouet C, Cha HR, Pang YY, Holmgren J, et al. Sublingual immunization with nonreplicating antigens induces antibody-forming cells and cytotoxic T cells in the female genital tract mucosa and protects against genital papillomavirus infection. J Immunol 2009; 183:7851-9; PMID:19933861; http://dx.doi.org/10.4049/jimmunol.0803740
40. Kildsgaard J, Brimnes J, Jacobi H, Lund K. Sublingual immunotherapy in sensitized mice. Ann Allergy Asthma Immunol 2007; 98:366-72; PMID:17458434; http://dx.doi.org/10.1016/S1081-1206(10)60884-8
41. Duc H, Hong HA, Fairweather N, Ricca E, Cutting SM. Bacterial spores as vaccine vehicles. Infect Immun 2003; 71:2810-8; PMID:12704155; http://dx.doi.org/10.1128/IAI.71.5.2810-2818.2003
42. Hoang TH, Hong HA, Clark GC, Titball RW, Cutting SM. Recombinant *B. subtilis* Expressing the Clostridium perfringens Alpha Toxoid Is a Candidate Orally Delivered Vaccine against Necrotic Enteritis. Infect Immun 2008 November 1, 2008; 76(11):5257-65.
43. Boucher P, Sato H, Sato Y, Locht C. Neutralizing antibodies and immunoprotection against pertussis and tetanus obtained by use of a recombinant pertussis toxin-tetanus toxin fusion protein. Infect Immun 1994; 62:449-56; PMID:7507893
44. Zhou Z, Xia H, Hu X, Huang Y, Li Y, Li L, et al. Oral administration of a Bacillus subtilis spore-based vaccine expressing Clonorchis sinensis tegumental protein 22.3 kDa confers protection against Clonorchis sinensis. Vaccine 2008; 26:1817-25; PMID:18329763; http://dx.doi.org/10.1016/j.vaccine.2008.02.015

45. Turnbull PC. Anthrax vaccines: past, present and future. Vaccine 1991; 9:533-9; PMID:1771966; http://dx.doi.org/10.1016/0264-410X(91)90237-Z
46. Hoa NT, Baccigalupi L, Huxham A, Smertenko A, Van PH, Ammendola S, et al. Characterization of Bacillus species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl Environ Microbiol 2000; 66:5241-7; PMID:11097897; http://dx.doi.org/10.1128/AEM.66.12.5241-5247.2000
47. Senesi S, Celandroni F, Tavanti A, Ghelardi E. Molecular characterization and identification of Bacillus clausii Strains marketed for use in oral bacteriotherapy. Appl Environ Microbiol 2001; 67:834-9; PMID:11157251; http://dx.doi.org/10.1128/AEM.67.2.834-839.2001
48. O'Hagan DT, MacKichan ML, Singh M. Recent developments in adjuvants for vaccines against infectious diseases. Biomol Eng 2001; 18:69-85; PMID:11566599; http://dx.doi.org/10.1016/S1389-0344(01)00101-0
49. Huang J-M, Hong HA, Van Tong H, Hoang TH, Brisson A, Cutting SM. Mucosal delivery of antigens using adsorption to bacterial spores. Vaccine 2010; 28:1021-30; PMID:19914191; http://dx.doi.org/10.1016/j.vaccine.2009.10.127
50. Wang Y, Zhang Z. [Bacterial spore–a new vaccine vehicle–a review]. Wei Sheng Wu Xue Bao 2008; 48:413-7; PMID:18479073
51. The Trouble in Tracing Opportunistic Pathogens. Cholangitis due to Bacillus in a French Hospital Caused by a Strain Related to an Italian Probiotic? Microb Ecol Health Dis 2000; 12:99-101; http://dx.doi.org/10.1080/089106000435491
52. Wang J, Fung DY. Alkaline-fermented foods: a review with emphasis on pidan fermentation. Crit Rev Microbiol 1996; 22:101-38; PMID:8817079; http://dx.doi.org/10.3109/10408419609106457
53. Raychaudhuri S, Rock KL. Fully mobilizing host defense: building better vaccines. Nat Biotechnol 1998; 16:1025-31; PMID:9831030; http://dx.doi.org/10.1038/3469
54. Simon O, Vahjen W, Taras D. Potential of probiotics in pig nutrition. Feed Mix (2007) 15, pp. 2-3. 34.
55. Hong HA, Duc H, Cutting SM. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev 2005; 29:813-35; PMID:16102604; http://dx.doi.org/10.1016/j.femsre.2004.12.001
56. Barnes AG, Cerovic V, Hobson PS, Klavinskis LS. Bacillus subtilis spores: a novel microparticle adjuvant which can instruct a balanced Th1 and Th2 immune response to specific antigen. Eur J Immunol 2007; 37:1538-47; PMID:17474150; http://dx.doi.org/10.1002/eji.200636875
57. Duc H, Hong HA, Fairweather N, Ricca E, Cutting SM. Bacterial spores as vaccine vehicles. Infect Immun 2003; 71:2810-8; PMID:12704155; http://dx.doi.org/10.1128/IAI.71.5.2810-2818.2003
58. Hoang TH, Hong HA, Clark GC, Titball RW, Cutting SM. Recombinant Bacillus subtilis expressing the Clostridium perfringens alpha toxoid is a candidate orally delivered vaccine against necrotic enteritis. Infect Immun 2008; 76:5257-65; PMID:18779344; http://dx.doi.org/10.1128/IAI.00686-08
59. Bumann D, Hueck C, Aebischer T, Meyer TF. Recombinant live Salmonella spp. for human vaccination against heterologous pathogens. FEMS Immunol Med Microbiol 2000; 27:357-64; PMID:10727892; http://dx.doi.org/10.1111/j.1574-695X.2000.tb01450.x
60. Casula G, Cutting SM. Bacillus probiotics: spore germination in the gastrointestinal tract. Appl Environ Microbiol 2002; 68:2344-52; PMID:11976107; http://dx.doi.org/10.1128/AEM.68.5.2344-2352.2002
61. Duc H, Hong HA, Cutting SM. Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. Vaccine 2003; 21:4215-24; PMID:14505901; http://dx.doi.org/10.1016/S0264-410X(03)00492-4
62. Driks A. Bacillus subtilis spore coat. Microbiol Mol Biol Rev 1999; 63:1-20; PMID:10066829
63. Rhee KJ, Sethupathi P, Driks A, Lanning DK, Knight KL. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J Immunol 2004; 172:1118-24; PMID:14707086
64. Isticato R, Cangiano G, Tran HT, Ciabattini A, Medaglini D, Oggioni MR, et al. Surface display of recombinant proteins on Bacillus subtilis spores. J Bacteriol 2001; 183:6294-301; PMID:11591673; http://dx.doi.org/10.1128/JB.183.21.6294-6301.2001
65. Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, et al. The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature 1997; 390:249-56; PMID:9384377; http://dx.doi.org/10.1038/36786
66. Medaglini D, Ciabattini A, Spinosa MR, Maggi T, Marcotte H, Oggioni MR, et al. Immunization with recombinant Streptococcus gordonii expressing tetanus toxin fragment C confers protection from lethal challenge in mice. Vaccine 2001; 19:1931-9; PMID:11228363; http://dx.doi.org/10.1016/S0264-410X(00)00434-5
67. Robinson K, Chamberlain LM, Schofield KM, Wells JM, Le Page RW. Oral vaccination of mice against tetanus with recombinant Lactococcus lactis. Nat Biotechnol 1997; 15:653-7; PMID:9219268; http://dx.doi.org/10.1038/nbt0797-653
68. Trollfors B, Knutsson N, Taranger J, Mark A, Bergfors E, Sundh V, et al. Diphtheria, tetanus and pertussis antibodies in 10-year-old children before and after a booster dose of three toxoids: implications for the timing of a booster dose. Eur J Pediatr 2006; 165:14-8; PMID:16249929; http://dx.doi.org/10.1007/s00431-005-1763-3
69. Rappuoli R, Pizza M, Douce G, Dougan G. Structure and mucosal adjuvanticity of cholera and Escherichia coli heat-labile enterotoxins. Immunol Today 1999; 20:493-500; PMID:10529776; http://dx.doi.org/10.1016/S0167-5699(99)01523-6
70. Ciabattini A, Parigi R, Isticato R, Oggioni MR, Pozzi G. Oral priming of mice by recombinant spores of Bacillus subtilis. Vaccine 2004; 22:4139-43; PMID:15474704; http://dx.doi.org/10.1016/j.vaccine.2004.05.001
71. Lee S, Belitsky BR, Brinker JP, Kerstein KO, Brown DW, Clements JD, et al. Development of a Bacillus subtilis-based rotavirus vaccine. Clin Vaccine Immunol 2010; 17:1647-55; PMID:20810679; http://dx.doi.org/10.1128/CVI.00135-10
72. Helting TB, Nau HH. Analysis of the immune response to papain digestion products of tetanus toxin. Acta Pathol Microbiol Immunol Scand C 1984; 92:59-63; PMID:6711309
73. Lavelle EC, O'Hagan DT. Delivery systems and adjuvants for oral vaccines. Expert Opin Drug Deliv 2006; 3:747-62; PMID:17076597; http://dx.doi.org/10.1517/17425247.3.6.747
74. Chadwick S, Kriegel C, Amiji M. Delivery strategies to enhance mucosal vaccination. Expert Opin Biol Ther 2009; 9:427-40; PMID:19344280; http://dx.doi.org/10.1517/14712590902849224
75. Mauriello EM, Duc H, Isticato R, Cangiano G, Hong HA, De Felice M, et al. Display of heterologous antigens on the Bacillus subtilis spore coat using CotC as a fusion partner. Vaccine 2004; 22:1177-87; PMID:15003646; http://dx.doi.org/10.1016/j.vaccine.2003.09.031
76. Byrne MP, Smith TJ, Montgomery VA, Smith LA. Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from Pichia pastoris as a recombinant vaccine candidate. Infect Immun 1998; 66:4817-22; PMID:9746584
77. Umland TC, Wingert LM, Swaminathan S, Furey WF, Schmidt JJ, Sax M. Structure of the receptor binding fragment HC of tetanus neurotoxin. Nat Struct Biol 1997; 4:788-92; PMID:9334741; http://dx.doi.org/10.1038/nsb1097-788
78. Emsley P, Fotinou C, Black I, Fairweather NF, Charles IG, Watts C, et al. The structures of the H(C) fragment of tetanus toxin with carbohydrate subunit complexes provide insight into ganglioside binding. J Biol Chem 2000; 275:8889-94; PMID:10722735; http://dx.doi.org/10.1074/jbc.275.12.8889
79. Lacy DB, Stevens RC. Sequence homology and structural analysis of the clostridial neurotoxins. J Mol Biol 1999; 291:1091-104; PMID:10518945; http://dx.doi.org/10.1006/jmbi.1999.2945