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Original article

Sublingual immunization with an engineered *Bacillus subtilis* strain expressing tetanus toxin fragment C induces systemic and mucosal immune responses in piglets

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Abstract

Sublingual (SL) and intranasal (IN) administration of a *Bacillus subtilis*-based tetanus vaccine was tested in piglets, which more closely mimic the human immune system than mice. Piglets were immunized by the SL, IN or oral routes with vaccine expressing tetanus toxin fragment C, or commercial tetanus vaccine given by intramuscular injection as a control. Tetanus toxoid specific ELISA and passive neutralization tests were used to measure IgG and IgA levels in serum and mucosal secretions, and assess protective serum antibodies, respectively. The nature of the immune response was explored by MHC Class II, TGF-β1 expression, and ELISA assays for multiple cytokines. SL or IN immunization of piglets induced neutralizing tetanus toxoid specific serum antibody and local salivary and vaginal IgA responses. Standard tetanus vaccine resulted in systemic antibodies, whereas oral administration of the *Bacillus*-based vaccine was ineffective. Further analyses indicated a balanced Th1/Th2 response to SL or IN immunization.

Conclusion: This study demonstrates for the first time that SL or IN administration is effective for inducing both systemic and mucosal responses in a piglet model, indicating that SL or IN delivery of a *B. subtilis*-based tetanus vaccine can be a simple, non-invasive, low cost strategy to induce immunity to tetanus.

Keywords: Piglets; Sublingual immunization; Intranasal immunization; *B. subtilis* bacteria; Tetanus vaccine

1. Introduction

The main point of entry for most microbial pathogens such as viruses, bacteria, parasites as well as environmental allergens are through mucosal surfaces. Therefore mucosal immune responses are known to function as a first line of defense [1]. The development of heat-stable vaccines with long shelf life that can be administered by mouth or directly to the nasal or sublingual mucosa would greatly facilitate immunization programs in resource poor developing countries by avoiding the need for a cold chain or injection. An additional advantage of mucosal vaccines is the potential to induce both local and systemic immune responses [2].

The mucosal immune system has developed various mechanisms to achieve and maintain tolerance against commensal bacteria, self-antigens and food [3].To prevent any detrimental response of the gastrointestinal tract to regular nutrients and commensal bacteria, the epithelial cells modify the stimulatory signals resulting in oral tolerance [4,5]. As a result, immunogens that induce vigorous immune responses if injected systemically do not generate any type of response if administered orally [6,7].

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Intranasal vaccination has been used in multiple species such as mice and monkeys, and previous work shows that IgA antibodies were detected in different regions of the body such as the gastrointestinal, respiratory and genital tracts [1,8–11]. Intranasal immunization is also known to stimulate the production of cytotoxic T lymphocytes (CTLs) in distant mucosal tissues [12], and greater systemic antibody responses compared to other mucosal immunization [1,8,11,13]. Levels of cytotoxic T lymphocytes and IgG antibodies obtained from mice and monkeys intranasally immunized with live virus are comparable to levels obtained from parenteral administration [13]. However, the lingering concern that live nasal vaccines may, in some instances, be transported in retrograde manner to the brain via olfactory nerves, potentially causing serious adverse events, is a rationale for seeking alternative mucosal immunization strategies [14]. Coupled with this is the fear that spores or vegetative cells might be able to replicate in the lung tissue.

Sublingual (SL) immunization is emerging as a novel alternative approach to mucosal vaccination against allergens and pathogens [15,16]. Live vaccine vectors and adjuvants that cannot be used orally or intranasally might be safe and effective if administered by the sublingual route. For example, papillomavirus vaccine administered via a SL route induced protective specific antibody and T cell responses in the genital tract, indicating the ability of SL immunization to induce an immune response at distant mucosal sites [17].

Bacillus subtilis cells and spores, present in widely consumed traditional Japanese or Korean foods made from soybeans fermented by addition of *B. subtilis* have an excellent safety record in humans. Several recent studies have shown that *B. subtilis* spores and cells engineered to express vaccine antigens can be used effectively to generate systemic and mucosal antibodies [11,18–22]. 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, when stored as lyophilized powders, have long-term stability during storage at elevated temperatures [11]. In mice, these vaccines are effective when administered either intranasally or sublingually [22]. In this study, we have evaluated the *B. subtilis* tetanus vaccine in piglets, and compared sublingual to intranasal, oral administration, and to the standard injectable DTaP vaccine.

2. Materials and methods

2.1. Preparation of spore-based and vegetative cell-based vaccines

Bacterial constructs used were described by Lee et al. [11]. Briefly, stable constructs of *B. subtilis* expressing TetC were created by integration of the tetanus toxin fragment coding sequence (here defined as TetC and corresponding to positions 2855 to 4237 of the tetanus toxin gene) into the bacterial chromosome. Antigen production was quantitated by immunoblotting [11].

Spores and vegetative cells were produced by growth in a defined medium without any animal-derived components [11]. Cells were harvested, washed and dried by speed vac. (2.5×10^9 cells per vial). On reconstitution with PBS, only ten percent of the cells were still viable after culture. The reconstituted cells were used for vaccination.

2.2. Piglet immunization

Five week-old weaned piglets were obtained from a commercial swine unit in MA. Five different groups of animals were immunized: 4 piglets received vegetative cells SL with mLT, 4 piglets received vegetative cells SL without mLT, 5 piglets received vegetative cells IN, 4 piglets received spores IN, 6 piglets received spores orally and 2 piglets received DTaP, the commercially available tetanus vaccine IM. DTaP and mucosally-immunized animals were housed and fed in separate rooms. Animals were immunized 4 times with 1×10^9 cells of dried TetC-expressing *B. subtilis* (BB3059 vegetative cells or BB3184 spores) reconstituted in PBS on days 0, 14, 28, and 42. Only 10 percent of the cells were viable at reconstitution, but vaccine dose was based on total number of cells. In some groups the cells were mixed with mutant heat labile toxin (mLT) of *Escherichia coli* as adjuvant. Comparison immunization animals received 250 ul of a commercial DTaP vaccine (Tripedia®, Sanofi Pasteur Inc., Swiftwater, PA, USA) IM on days 0, 14, and 28.

For SL immunization, animals were anesthetized with ketamine (100 mg/kg of bodyweight) and xylazine hydrochloride (10 mg/kg of bodyweight), and positioned sternal side down. One ml of vaccine containing 1×10^9 cells of dried TetC-expressing *B. subtilis* reconstituted in PBS was slowly placed under the tongue with a tuberculin syringe. Animals remained recumbent for 30 min. For IN immunization, 500 ul of solution containing 1×10^9 cells of dried TetC-expressing *B. subtilis* vegetative cells (BB3059) or spores (BB3184) were placed into each nostril. Oral inoculation was achieved by gavage with a total volume of 5 ml containing 1×10^9 vegetative cells or spores. Control piglets received non TetC-expressing *B. subtilis vegetative cells* alone.

2.3. Sample collection and colonization/shedding assay

Blood was collected via the jugular vein, centrifuged, separated and serum stored at −20 °C. Saliva was collected by inserting cotton swabs under the tongue of the anesthetized pigs, incubated for 1 h at room temperature (RT) in 70 μl of PBS containing 0.1% BSA, 0.05% Tween 20.Vaginal wash was collected by flushing with 50 μl of PBS. Fecal samples were manually collected from piglets and frozen at −20 °C. Ten percent fecal suspensions were prepared and diluted in PBS containing 0.05% Tween 20, 1% BSA. For culture, 10% suspensions were prepared in DS medium plus 15% glycerol and stored at −20C until cultured on DS agar plates containing kanamycin at 5 ug/ml. All samples were centrifuged (13,800 g for 10 min), and supernatants were used for analysis. Blood and fecal samples were collected prior to each immunization and two weeks after the last (fourth) immunization. Saliva and vaginal washes were collected two weeks