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Pollen grains for oral vaccination

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ABSTRACT

Oral vaccination can offer a painless and convenient method of vaccination. Furthermore, in addition to systemic immunity it has potential to stimulate mucosal immunity through antigen-processing by the gut-associated lymphoid tissues. In this study we propose the concept that pollen grains can be engineered for use as a simple modular system for oral vaccination. We demonstrate feasibility of this concept by using spores of *Lycopodium clavatum* (clubmoss) (LSs). We show that LSs can be chemically cleaned to remove native proteins to create intact clean hollow LS shells. Empty pollen shells were successfully filled with molecules of different sizes demonstrating their potential to be broadly applicable as a vaccination system. Using ovalbumin (OVA) as a model antigen, LSs formulated with OVA were orally fed to mice. LSs stimulated significantly higher anti-OVA serum IgG and fecal IgA antibodies compared to those induced by use of cholera toxin as a positive-control adjuvant. The antibody response was not affected by pre-neutralization of the stomach acid, and persisted for up to 7 months. Confocal microscopy revealed that LSs can translocate into mouse intestinal wall. Overall, this study lays the foundation of using LSs as a novel approach for oral vaccination.

1. Introduction

Oral vaccination is of significant interest because it is needle-free, painless, child-friendly, convenient, and amenable to self-administration. Furthermore, antigen delivery to the gastrointestinal tract has potential to induce mucosal immunity through antigen-processing via the gut-associated lymphoid tissues [1,2]. Because majority of pathogens infect via mucosal surfaces, immunological defenses at mucosal surfaces can neutralize pathogens before they can cause infection [3].

However, oral vaccination remains daunting because the vaccine molecules experience a highly degradative environment in the stomach, and encounter a tough transport barrier offered by the tightly juxtaposed epithelial cells that line the intestinal mucosa [4]. To overcome these delivery challenges, live attenuated microorganisms such as bacteria and viruses, and particulate vaccine delivery systems such as liposomes, virosomes, polymeric micro and nanoparticles, and immune stimulating complexes have been examined [5,6]. Various techniques that seek to target microfold (M) cells to enable higher antigen uptake through endocytosis have also been tested [7]. Toxins such as cholera toxin (CT) and heat labile enterotoxin (LT) that enable increased uptake of vaccine molecules have demonstrated the highest adjuvant effects [8,9]. However, the toxicity of CT and LT naturally renders them unsuitable for human use [10]. The risk of attenuated yet live strains of viruses and bacteria to become virulent, or their neutralization through pre-existing host immunity are some of the limitations of using microorganisms for oral vaccination [11]. Additionally, low encapsulation efficiency of antigens in polymeric particles, and poor vaccine stability resulting from exposure to organic solvents during particle synthesis have hindered clinical use of polymeric particles [12]. Plant-based edible vaccines expressed in rice [13], tobacco, tomato, carrot and potato have also been investigated [14]. Based on a 20-year development experience of an edible vaccine against hepatitis B, it has been found that just oral administration of edible plant-based hepatitis vaccine was insufficient for a protective response, yet when coupled in an oral-parenteral dosage regimen, it proved to be effective [14].

In this study we propose a novel concept to use pollen grains (PGs) as a natural system for oral vaccination. In nature, PGs facilitate pollination by functioning as a 'safety-pod' to carry the plant male gamete to the female reproductive organ, the ovary, located in the flower. During pollination PGs are often exposed to fluctuating temperatures and weather conditions, and to survive such conditions, they possess mechanical toughness and chemical resistance. Our postulate to use PGs for oral vaccination is based on such multiple natural properties of PGs that suggest their usefulness for oral vaccination: (i) they have a tough outer shell that can stay intact in the stomach environment [15,16], (ii) the shell is naturally porous, and (iii) despite their relatively large size (tens of micrometers in diameter) it has been found that *Lycopodium clavatum* (clubmoss) spores (LSs) and *Secale cereale* (rye) pollen grains can cross the intestinal barrier as intact particles [17,18]. Thus overall, we hypothesized that if (i) natural pores in the pollen wall could be used to clean and remove the allergy-causing native biomolecules from PGs, (ii) their clean 'belly'

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could be refilled with vaccine antigens through the natural pores in pollen walls, and (iii) the antigen-filled PGs could translocate across the intestinal epithelium into the body, then PGs might behave as natural 'Trojan horses' for oral vaccination ferrying the vaccines safely into the body. While LSs can survive the harsh acidic treatment, it has been suggested that enzymes in the body can degrade them [18,19], thus providing a potentially safe natural carrier for oral vaccine and drug delivery. Indeed, using this conceptual framework LSs have recently been proposed for oral drug delivery. It has been shown that proteins as large as 540 kDa, a magnetic resonance imaging contrast agent, food oils including cod liver oil can be filled into LSs [19–23]. While these in vitro studies demonstrate the flexibility of filling LSs with different molecules, in vivo demonstrations on the effectiveness of pollens for oral drug and vaccine delivery are lacking.

Herein we demonstrate for the first time that LSs filled with ovalbumin (OVA) as a model antigen when fed orally to mice can induce a systemic and a mucosal immune response, which is superior to that stimulated by CT, a potent yet toxic mucosal adjuvant. We also investigated whether neutralization of stomach's acidic environment, prior to administration of the LS-based oral vaccine can affect the immune response. The durability of antigen-specific systemic and mucosal antibodies was also investigated, and it was found that OVA-specific antibodies could be detected in significant amounts up to 7 months after vaccination. Overall this study lays the foundation for an oral vaccination platform that is simple to implement and has potential for applicability to a broad range of vaccines.

2. Materials and methods

2.1. Pollens, chemicals, proteins and antibodies

LSs, dextran conjugated to fluorescein isothiocyanate (4000 Da and 2000 kDa), sulforhodamine (558 Da), and phosphate-citrate buffer tablets were purchased from Sigma-Aldrich (MO, USA). Pollens of *chenopodium album* (lambs quarters), *helianthus annuus* (sunflower), *artemisia vulgaris* (mugwort), and *alnus glutinosa* (alder black) were obtained from Pharmallerga (Lišov, Czech Republic). Acetone, potassium hydroxide, orthophosphoric acid, ethanol, hydrochloric acid, sodium hydroxide and tween 20 were purchased from Fisher Scientific (PA, USA). O-phenylenediamine (OPD) was obtained from Invitrogen (NY, USA). Milli-Q water with a resistance of 18.2 MΩ cm was used in all experiments. OVA was purchased from MP Biomedicals (OH, USA). CT and CTB were purchased from Sigma-Aldrich (MO, USA). Goat anti-mouse IgG, IgG1, IgG2a, IgA, and IgE with the horseradish peroxidase (HRP) conjugate were bought from Southern Biotech (AL, USA). Texas-red labeled OVA and bovine serum albumin were purchased from Invitrogen (OR, USA).

2.2. LS treatment and characterization

LSs were chemically treated to produce intact clean spores by modifying a previously published process [24]. Briefly, 50 g of dry LSs were stirred in 300 mL of acetone under reflux overnight. Following filtration and overnight drying, they were stirred under reflux in 450 mL of 2 M potassium hydroxide for 12 h at 120 °C (renewed after 6 h). They were then filtered and washed with hot water (5 × 300 mL) and hot ethanol (5 × 300 mL). After drying overnight, LSs were stirred under reflux for 7 days in 450 mL of orthophosphoric acid at 180 °C. LS were filtered and washed sequentially with water (5 × 300 mL), acetone (300 mL), 2 M HCl (300 mL), 2 M NaOH (300 mL), water (5 × 300 mL), acetone (300 mL) and ethanol (300 mL). Finally, they were dried at 60 °C until constant weight was achieved. The final protein concentration of the LSs was measured using nitrogen elemental analysis (PerkinElmer 2400 Series II CHNS/O Analyzer), which measures percent nitrogen in the sample. A multiplication factor of 6.25 was used to convert percent nitrogen to percent protein [25]. Scanning electron micrographs (SEM) of LSs before and after treatment were taken to confirm the removal of the biomolecules, and to determine if chemical treatment causes any damage to LSs.

2.3. Filling LSs with foreign molecules

To assess the ability to fill different molecules into LS core, dry chemically treated LSs were added to respective aqueous solutions of sulforhodamine, dextran conjugated to fluorescein isothiocyanate, ovalbumin conjugated to Texas red, or bovine serum albumin conjugated to Texas red. Vacuum of about 25 in. of Hg was applied overnight by placing the aqueous suspensions of LSs in a vacuum chamber. LSs were then imaged using confocal microscopy (Ti-E inverted microscope with C2+ point scanning confocal system, Nikon Melville, NY).

2.4. Vaccine formulations

Vaccine formulations were prepared as follows in 0.3 mL PBS (dose per mouse): *OVA alone*: OVA(100 μg) — dose was selected based on a previous study [26]; *LS1*: OVA(100 μg) + LS(1 mg); *LS5*: OVA(100 μg) + LS(5 mg); *CT1*: OVA(100 μg) + CT(5 μg); *CT2*: OVA(5 mg) + CT(5 μg). Higher dose of OVA was used because it has been shown that CT is more effective when OVA dose is high [26]; *CTB:* OVA(100 μg) + CTB(50 μg). B-subunit of CT is a safer mucosal adjuvant than CT, but it is less immunogenic.

2.5. Immunizations and sample collection

BALB/c female mice 6–8 weeks old were purchased from Charles River Laboratories (MA, USA) and were maintained at Texas Tech University Animal Care Services (TX, USA). All treatments were performed according to Texas Tech Animal Care and Use Committee (IACUC) approved procedures. All mice were fed an OVA-free diet. Mice ($n = 5$ per group) were orally immunized by administering each mouse 0.3 mL of the vaccine formulation (OVA alone, CT1, CT2, CTB, LS1 or LS5) using a 27G blunt-tipped feeding needle. In a separate experiment, to examine the effect of neutralization of gastric acid prior to oral vaccination, mice were deprived of food and water 2 h prior to the immunizations. Next they were given an oral gavage of 0.3 mL of sodium bicarbonate (8 parts PBS + 2 parts 0.34 M sodium bicarbonate) [27,28] to neutralize the acid in their stomach, and 30 min later were orally immunized with OVA alone (*OVA-Na group*) or LS5 formulation (*LS5-Na group*). Separate groups of mice also received OVA alone or LS5 without pre-feeding with sodium bicarbonate. In all experiments, mice were immunized on days 0 and 28. Blood was collected from animals via the retro-orbital plexus on days 0, 28 and 56. Sera were stored at −20 °C until analysis. For the determination of mucosal immune response about 10–15 fecal droppings were collected from each animal, processed in PBS, and the saline extracts were stored at −20 °C until analysis. For measuring the long term immune response, in some groups serum and fecal matter were also collected at 7 months after first oral dose (day 0).

2.6. Antibody measurement

OVA-specific antibodies in (i) pooled sera diluted from 1:50 to 1:6400, (ii) individual mouse sera at a dilution of 1:200, 1:400, or 1:25, and (iii) fecal samples at an individual dilution of 1:5 were analyzed by standard ELISA. The wells of a 96-well plate (Maxisorp-Nunc, Sigma-Aldrich, MO, USA) were coated with 50 μL of 5 μg/mL OVA solution in PBS and kept overnight at 4 °C. The wells were then blocked using 100 μL of milk (5% in PBS containing 0.05% tween 20) for 1.5 h at room temperature. Pooled serum samples from each group were serially diluted (from 1:50 to 1:6400), added (50 μL) in the wells of the plate, and incubated at room temperature for 1.5 h. Secondary