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Bacterial spores as particulate carriers for *gene gun* delivery of plasmid DNA

Luana R.M.M. Aps[a], Milene B. Tavares[a], Julio H.K. Rozenfeld[b], M. Teresa Lamy[b], Luís C.S. Ferreira[a], Mariana O. Diniz[a,*]

[a] *Vaccine Development Laboratory, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil*
[b] *Institute of Physics, University of São Paulo, Brazil*

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ABSTRACT

Bacillus subtilis spores represent a suitable platform for the adsorption of proteins, enzymes and viral particles at physiological conditions. In the present work, we demonstrate that purified spores can also adsorb DNA on their surface after treatment with cationic molecules. In addition, we demonstrate that DNA-coated *B. subtilis* spores can be used as particulate carriers and act as an alternative to gold microparticles for the biolistic (*gene gun*) administration of plasmid DNA in mice. Gene gun delivery of spores pre-treated with DODAB (dioctadecyldimethylammonium bromide) allowed efficient plasmid DNA absorption and induced protein expression levels similar to those obtained with gold microparticles. More importantly, we demonstrated that a DNA vaccine adsorbed on spores can be loaded into biolistic cartridges and efficiently delivered into mice, which induced specific cellular and antibody responses. Altogether, these data indicate that *B. subtilis* spores represent a simple and low cost alternative for the in vivo delivery of DNA vaccines by the gene gun technology.

1. Introduction

The functionality of DNA-based vaccines relies on the *in vivo* transfection of cells that express the encoded antigens and, subsequently, lead to activation of antigen presenting cells (APC) that deploy innate and adaptive immune responses (Kutzler and Weiner, 2008; Nguyen et al., 2008). Therefore, experimental strategies to enhance cell transfection efficiency and, consequently, the potency of the induced immune responses are of great relevance. In this context, micro- and nanoparticles carriers have been widely used for the improvement of DNA vaccine strategies as an attempt to overcome their low immunogenicity, particularly in larger mammalian species, such as non-human primates and humans (O'Hagan et al., 2001).

Biolistic administration represents one alternative to increase the performance of DNA vaccines by increasing the number of antigen-transfected cells. This administration method, also known as *gene gun* delivery, introduces the plasmid DNA directly into the epidermis, an anatomical site rich in APCs, particularly Langerhans cells (LHC, immature dendritic cells). Gene gun immunization promotes enhanced DNA transfection and antigen expression leading to DC maturation and improved priming and activation of effector T cell responses (Lin et al., 2010). As a consequence, biolistic immunization requires much lower DNA amounts compared to intradermal or intramuscular immunizations to induce similar immune responses (Rezvan et al., 2011; Nguyen-Hoai et al., 2012; Ginsberg et al., 2010; Kim et al., 2004).

Gene gun delivery uses compressed gas to propel micrometer-sized gold particles coated with plasmid DNA (O'Brien and Lummis, 2006; Woods and Zito, 2008; Gotesman and Williams, 2016). Cationic agents (spermidine and calcium) are used to generate positive charges on the gold particle surface, enabling them to interact electrostatically with the negatively charged nucleic acid molecules. This technology is easily handled and has been largely employed to induce gene expression for different goals in plants (Klein et al., 1988), animals (De Rose et al., 2002; Lambracht-Washington et al., 2011; Nguyen-Hoai et al., 2015) and humans (Sidney, 2016). Several studies have been performed with an optimized process to improve DNA loading and the use of biodegradable and less expensive particles (Svarovsky et al., 2008; Kasturi et al., 2006).

Bacterial spores have different applications in biotechnology and vaccinology (Ricca and Cutting, 2003). The spores of some *Bacillus* sp. strains interact with heavy metals, such as Cd, Zn,

* Corresponding author at: Av. Prof. Lineu Prestes, 1374 São Paulo, SP, Brazil.
E-mail address: modiniz@usp.br (M.O. Diniz).

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Mn(II), Cu(II) and Ni (Tebo, 1995; He and Tebo, 1998; Hinc et al., 2010), standing out in ecosystem bioremediation research. Moreover, recent studies demonstrated that *B. subtilis* spores have a negatively charged surface, consisting of a suitable platform to adsorb positively charged molecules, including proteins (Huang et al., 2010), viral particles (Song et al., 2012) and enzymes (Sirec et al., 2012). Additionally, *B. subtilis* spores are safe to handle and their production and purification procedures are simple, quick and inexpensive (Tavares et al., 2013).

In this work, we demonstrated that plasmid DNA can be easily adsorbed on the surface of *B. subtilis* spores after a single step procedure based on the use of cationic agents, particularly dioctadecyldimethylammonium bromide (DODAB). DODAB cationic lipid and its assemblies have been employed in distinct contexts, such as imaging, biosensing, gene and drug delivery and vaccines (Carmona-Ribeiro, 2010). Sonication of DODAB vesicles in water produces small bilayer fragments that have previously been used as antimicrobial agents or as immunoadjuvant carriers for proteins (Rozenfeld et al., 2012). These fragments can activate dendritic cells and stimulate antigen presentation (Lincopan et al., 2009). In this study, we showed that DNA-coated spores can be loaded into gene gun cartridges and efficiently employed to transfect cells under *in vitro* and *in vivo* conditions. In addition, mice immunized with spores loaded with a DNA vaccine showed enhanced antigen-specific cellular and humoral immune responses at similar or higher levels to those achieved by gold microparticles, thus representing a low cost alternative to be used in gene delivery.

2. Material and methods

2.1. Mice

Male C57BL/6 mice at 6–8 weeks of age were supplied by the Animal Breeding Center of the Biomedical Sciences Institute of the University of São Paulo and housed at the Parasitology Department of the University of São Paulo. All the procedures involving animal handling followed the recommendations for the proper use and care of laboratory animals from the University of São Paulo Ethics Committee (protocol number 95-2011).

2.2. DNA plasmid

The pLuc plasmid encodes the luciferase enzyme (*luc2* gene). The gene sequence was cloned in the pcDNA3.0 vector (Invitrogen, CA, USA), which contains a CMV promoter and an ampicillin/neomycin-resistance-encoding gene. The DNA vaccine pgDE7 h encodes the HPV-16 E7 oncoprotein genetically fused near the C-terminal portion of the HSV-1 gD protein, as previously described (Diniz et al., 2013). The chimeric gene sequence was cloned in the pUMVC3 vector (Aldevron, ND, USA), which contains a CMV promoter and a kanamycin-resistance-encoding gene.

2.3. Preparation of spores

The sporulation of *B. subtilis* strain 1012 was performed by the nutrient exhaustion method using the Foerster medium (Foerster and Foster, 1966) and adapted from work by (Tavares et al., 2013). After 7 days of sporulation, the cultures were centrifuged at 10,000 rpm for 10 min and washed three times with distilled water. The spores were suspended in water and incubated for 1 h at 68 °C before quantification. The spores were visualized under a digital microscope EVOS® (AMG) and titrated to determine the number of colony-forming units (CFU/ml), confirmed by Petroff-Hausser chamber quantification (spores/ml). Heat-killed spores were obtained after autoclaving (121 °C for 15 min).

2.4. Particle size and zeta potential assays

Particle size measurements were determined from the mean hydrodynamic diameter. Zeta potentials (ζ) were determined from the electrophoretic mobility μ and the Smoluchowski equation, $\zeta = \mu\eta/\varepsilon$, where η and ε are the viscosity and the dielectric constant of the medium, respectively. Particle size and zeta potential measurements were evaluated before or after adsorption of pDNA to spore surfaces. Each spore sample was concentrated by centrifugation, suspended in 1 ml of water and measured three times with 10 readings for each measurement. Size distribution, zeta-average diameter and zeta-potential for all dispersions were determined by the dynamic light scattering (DLS) technique as described by (Rozenfeld et al., 2012) using a ZetaSizer NanoZS90 Analyzer (Malvern Instruments Ltd., Worcestershire, UK).

2.5. Adsorption of DNA to spores

B. subtilis spores were tested for the ability to adsorb plasmid DNA with different cationic agents. Spores (5×10^8) were suspended in 1 ml of each reagent listed in Table 1 and incubated at room temperature for 1 h with stirring every 15 min. After incubation, the suspension was centrifuged (10,000 rpm/10 min) and the pellet was washed once with distilled water. The spore samples were suspended in 100 μl of a solution containing 1 μg of plasmid DNA according to the conditions described in Table 1. After centrifugation, non-adsorbed DNA was estimated in agarose gels using HindIII-digested λ DNA markers. For biolistic immunization, the adsorption was performed using spores, pDNA and reagent amounts proportionally to the procedure described above.

2.6. Cartridge preparation for gene gun

The spores with adsorbed plasmid DNA were loaded on gene gun cartridges for biolistic administration. First, spores were suspended in a 0.05 mg/ml PVP (polyvinylpyrrolidone) in 100% ethanol and laid in Tefzel® tubes (BioRad) appropriate for the gene gun cartridge preparation. The tubes remained under airtight conditions overnight. Then, after removal of the liquid, the spores bound at the inner tube surface were dried with helium gas. To facilitate the administration, we used 2-fold amounts of pDNA and microparticles per cartridge. For both plasmid DNAs (pLuc and pgDE7 h), adsorption was carried out with 10 mg (1×10^{10}) of spores or gold for the preparation of 10 cartridges containing 2 μg of pLuc or pgDE7 h per cartridge (totalizing 20 μg of pDNA per tube). The cartridges were stored at −20 °C until use. The gold microparticle cartridges were prepared according to the manufacturerís instructions (BioRad) and stored at room temperature.

2.7. In vitro and in vivo gene gun transfection using a luciferase gene reporter

COS-7 cells were cultured in RPMI medium containing 10% (v/v) fetal bovine serum (FBS) and kept at 37 °C at 5% CO_2. Cells were seeded in 60 mm^2 culture plates (10^6 cells per plate) and incubated until 80% confluence. After medium removal, cells were transfected with 1 μg of pLuc using the a biolistic helium particle accelerator (Biomics, Brasília, Brazil) (100 psi of pressure) positioned 3 cm away from the cell plate, followed by the addition of 5 ml of RPMI with 2% FBS. C57BL/6 mice were inoculated using the gene gun device at 500 psi of pressure with 2 μg or 4 μg of pLuc on the shaved abdominal skin. Bioluminescence measurements were carried out 48 h after transfection following incubation for 20 min with 150 μg/ml luciferin solution in the cell plates or 20 min after intraperitoneal injection of 150 mg/kg of body weight of luciferin solution. Image captures and quantification analyses were performed using IVIS®