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


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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.

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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 Lumnis, 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,
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 (Alderlon, 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, \( \zeta = \frac{\mu \eta}{\varepsilon} \), 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 × 10^10) 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 biologic 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 biologic 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 manufacturer’s 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% fetal bovine serum (FBS) and kept at 37 °C at 5% CO2. Cells were seeded in 60 mm² 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 biologic helium particle accelerator (Biomics, Brasilia, 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®.
 Spectrum equipment (Caliper). The bioluminescence images were shown in the “photon” mode and subsequently analyzed on the same equipment to obtain the total flux (photons/sec) or the radiance values (pJ/sec/cm²/sr), which refers to the number of photons per second per tissue area in a solid angle (“stereadian”, “sr”).

2.8. Immunization with a DNA vaccine using a gene gun

C57BL/6 were immunized twice (14 day interval) with pgDE7·h, which encodes the type 16 human papillomavirus (HPV-16) E7 oncoprotein genetically fused to the D glycoprotein of the type 1 human herpes virus (HSV) (Diniz et al., 2013; Aps et al., 2015). Gene gun cartridges were prepared with spores or gold microparticles containing either 4 μg or 2 μg of plasmid DNA, respectively. The shots were applied in the shaved abdominal region, with the equipment touching the skin of the mice. Blood samples were collected two weeks after the first and second dose for antibody and CD8+ T lymphocyte assay. Mice treated with a gene gun with empty cartridges were considered as the negative control group and an additional control group vaccinated with non-coated spores (spores treated with DODAB but not incubated with DNA) was used following the same immunization regimen.

2.9. Enzyme-Linked immunosorbent assay (ELISA)

The anti-E7 and anti-gD antibody titers were measured by ELISA, as previously described (Sbrogi-Almeida et al., 2004). Plates (Polysorp™, Nunc-Immuno plates) were coated with 250 ng/well of gD recombinant protein obtained as previously described by Porchia and colleagues (Porchia et al., 2011). After incubation for 16 h at 4°C, the plates were blocked with 3% gelatin in PBS and incubated at 37°C for 2 h. Samples of individual sera collected 14 days after the first and second doses were added at a 1:250 dilution and incubated at 37°C for 1 h. After incubation, anti-mouse IgG (1:3000), IgG1 (1:10,000) or IgG2c (1:3000) conjugated to peroxidase was added and the reaction was later developed with a solution containing o-phenylenediamine dihydrochloride (OPD). The optical densities were determined at 492 nm in an Epoch™ Multi-Volume Spectrophotometer (Bio Tek Instruments) All samples were assayed in duplicate and the absorbance values of the control reactions (“blank”, wells without serum addition) were subtracted from the results obtained with the tested serum samples. Dilution curves were drawn for each sample and the titers calculated as the reciprocal values of the highest dilution with an optical density of 0.2 (established as the value between the mean OD of the blank ±3 standard deviations).

2.10. Intracellular cytokine staining (ICS)

Intracellular IFN-γ staining was performed using blood samples collected 14, 21 or 28 days after the vaccine administration, according to previously described procedures (Diniz et al., 2013). The blood samples were treated for lysis of red blood cells and cultured at a concentration of 10⁶ cells/well for 6 h at 37°C in 96-well round bottom microtiter plates with 10 μg/ml of Brefeldin A ( GolgiPlug; BD Biosciences, CA, USA) in the presence or not of 3 μg/ml of the E7-specific RAHYNIVTF peptide (amino acids 49–57). After incubation, the cells were stained with FITC-conjugated anti-CD8a antibody and after fixation and permeabilization, with PE-labeled anti-IFN-γ. The buffers and antibodies were purchased from BD Biosciences (CA, USA). The cells were examined by flow cytometry using a FACS Fortessa (BD Biosciences) and the data were analyzed using FlowJo software (TreeStar, OR, USA).

2.11. Statistical analyses

The ANOVA test was applied followed by Tukey’s post-test when two or more groups were compared. Student’s t-test was applied for analyses comparing individual data points from two groups. GraphPad Prism software was used for both analyses.

3. Results

3.1. Adsorption of plasmid DNA on the surface of spores

The adsorption of molecules on B. subtilis spores has been achieved with a variety of molecules, including antigens, biologically active enzymes and whole viral particles. In this study, we aimed to test whether these bioparticles can adsorb a DNA plasmid and deliver it to eukaryotic cells. First, we developed an adsorption procedure using different cationic reagents: spermine, calcium chloride (CaCl₂), cetyltrimethylammonium bromide (CTAB), and bilayer fragments of dioctadecyldimethylammonium bromide (DODAB BF) at different concentrations and conditions (Table 1). Treatment of live spores (5 x 10⁶) with the different chemical reagents was performed for 1 h at room temperature, and different incubation periods and temperatures for DNA binding. The adsorption efficiencies were determined by measuring the amount of DNA in the supernatant of the spore-DNA suspension after centrifugation.

Sporo-treated with 0.05 M spermine and 0.01% CTAB partially adsorbed DNA after incubation for 1 h at 25°C. No significant improvement was obtained after a prolonged incubation (6 h) at 25°C or 4°C. In contrast, the adsorption of DNA to spores treated with 2 mM DODAB was faster and more efficient. Using this reagent, more than 90% of the total DNA bound to the spores after incubation at 25°C or immediately after mixing (termed “no incubation”). On the other hand, the incubation of spores at pH 4 or pH 7, used previously for protein adsorption (Huang et al., 2010), did not result in significant binding of DNA on the spore surface, nor did incubating the spores with 1 M CaCl₂. The same results were obtained with heat-inactivated spores (data not shown). In conclusion, the adsorption of plasmid DNA on spores treated with 2 mM DODAB was superior to all other tested conditions. Further experiments were carried out using DODAB-treated spores and DNA adsorption for 1 h at 25°C.
3.2. Biophysical characterization of spores adsorbed with DNA

After determining the optimal conditions to adsorb DNA on spores, we next directed our study to investigate biophysical parameters involved in the reaction, such as the size and charge profiles. Purified live or heat inactivated spores of the B. subtilis 1012 strain had negative zeta potentials (LS: $-35\,\text{mV} \pm 0.8$; KS: $-30\,\text{mV} \pm 0.4$). Treatment with DODAB caused a sharp shift in the spore zeta potential to positive (LS: $+33\,\text{mV} \pm 0.2$; KS: $+29\,\text{mV} \pm 0.7$). After the addition of DNA, the zeta potentials of both live and inactivated spores shifted again and became close to neutral (LS: $8\,\text{mV} \pm 0.9$; KS: $-4\,\text{mV} \pm 0.1$) (Fig. 2A), providing evidence for the binding of the plasmid DNA on the spore surface.

In addition, the size (Fig. 2B–C) and polydispersity (Pdl) (Table 2) of spore-DODAB-DNA complexes were assessed by the DLS technique. Live and heat-inactivated spores had an average diameter of approximately 1.0 $\mu$m and presented similar Pdl, approximately 0.16. After treatment with DODAB and DNA, the spore sizes did not change significantly compared to untreated spores, showing no significant difference between these samples (Fig. 2B–C). Nonetheless, DNA coating resulted in a very polydispersed system, with PDI $= 1$ (Table 2).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Polydispersity index of B. subtilis spores during the adsorption steps.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microparticle</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>LS</td>
<td>$0.163 \pm 0.096$</td>
</tr>
<tr>
<td>LS-DODAB</td>
<td>$0.348 \pm 0.041$</td>
</tr>
<tr>
<td>LS-DODAB-DNA</td>
<td>$0.537 \pm 0.153$</td>
</tr>
<tr>
<td>KS</td>
<td>$0.160 \pm 0.033$</td>
</tr>
<tr>
<td>KS-DODAB</td>
<td>$0.805 \pm 0.191$</td>
</tr>
<tr>
<td>KS-DODAB-DNA</td>
<td>$1.000 \pm 0.000$</td>
</tr>
</tbody>
</table>

Next, we determined the minimal amount of spores required to adsorb $1\,\mu$g of plasmid DNA. For that purpose, different spore amounts ($10^7$–$5 \times 10^8$ spores) were incubated with $1\,\mu$g of pLuc for 1 h at 25°C (Fig. 1). Complete adsorption of DNA was achieved with $5 \times 10^8$ spores, corresponding to 0.5 mg of spore dry mass (Fig. 1A). The same results were also obtained with linear DNA fragments (synthetic oligonucleotides of 21 mer) (Fig. 1B), with an adsorption rate of $1\,\mu$g to $5 \times 10^8$ spores.
3.3. Spores as microparticles for gene gun-mediated transfection of eukaryotic cells

To investigate the use of B. subtilis spores as a platform for biolistic administration of plasmid DNA, we first determined the transfection efficiencies in COS-7 cells cultured with a luciferase-encoding plasmid (pLuc). As indicated in Fig. 3, cells submitted to biolistic administration with gold particles were efficiently transfected with pLuc. Similarly, biolistic administration using heat-inactivated spores had similar emission results, with no statistical difference in regard to gold group but a higher luminescence emission compared to live spores (approximately 7-fold) after 24 h (Fig. 3B), indicating that under the tested parameters, inactivated spores can be used for transfection of eukaryotic cells using gene gun delivery of plasmid DNA.

3.4. Spores as microparticles for in vivo delivery of plasmid DNA

Next, we evaluated if DNA-loaded B. subtilis spores could promote in vivo transfection of mice after gene gun administration. C57BL/6 mice were inoculated in the abdominal region with pLuc adsorbed on spores (2 or 4 µg/dose) or gold microparticles (2 µg/dose). After 48 h, mice were evaluated for bioluminescence emission using the substrate luciferin (Fig. 4). Using the same amount of DNA for both spores and gold microparticles (one shot of 2 µg of pLuc), live (LS) or killed spores (KS) promoted half the luminescence emission as gold microparticles (Fig. 4B). Notably, after two shots of DNA-coated spores (4 µg of pLuc), killed spores showed similar protein expression levels compared to gold microparticles coated with 2 µg of pLuc, suggesting that heat-inactivated spores could be a suitable carrier for the delivery of DNA vaccines by gene gun devices. The luminescence in mice inoculated with live spores was significantly lower than in animals inoculated with heat-inactivated spores, as previously observed in the in vitro assays.

3.5. Detection of immune responses elicited in mice immunized by gene gun with heat-inactivated subtilis spores

C57BL/6 mice were immunized with spores loaded (4 µg/dose) with a plasmid named pgDE7 h, encoding a chimeric protein derived from the human papillomavirus E7 oncoprotein genetically fused to the herpes virus glycoprotein D. Intramuscular immunization of mice with the same amount of naked DNA did not induce a significant E7-specific CD8+ T cell activation (Diniz et al., 2013) or serum IgG response to the HSV-1 gD protein (Aps et al., 2015). Mice immunized with DNA adsorbed on inactivated B. subtilis spores induced the activation of T CD8+ IFN-γ+ cells targeting the E7 oncoprotein of HPV-16 at numbers equivalent to those achieved in mice immunized with gold particles (Fig. 5A). In addition, mice immunized with pgDE7 h adsorbed on spores showed a high antibody response for the HSV-1 gD component of the hybrid protein at
levels similar to those observed in animals immunized with gold particles (Fig. 5B). In addition, gene gun vaccination using spore or gold microcarriers promoted a balanced Th1 and Th2 response with a slight trend to the Th2 profile (Fig. 5C). Collectively, these data show that killed B. subtilis spores can be used as an alternative for gold microparticle for the in vivo gene gun delivery of DNA vaccines.

4. Discussion

Recently, B. subtilis spores have been tested as a platform for loading different molecules with various biotechnological applications. However, to our knowledge, no study has investigated spores coated with plasmid DNA or assessed the potential application of spores as a particulate support for the gene gun delivery of DNA. In this work, we demonstrated that B. subtilis spores can adsorb DNA plasmids after a simple procedure using vesicular fragments of DODAB. In addition, our work showed that heat-killed spores can be loaded into biologic cartridges for gene gun administration and promote in vitro and in vivo transfections, also inducing specific immune response as achieved with gold microparticles.

In agreement with previous reports (Huang et al., 2010; Kazakov et al., 2008), our work showed that live and heat-killed spores were approximately 1 μm in diameter and had a negative surface charge (Fig. 2), which is not compatible with the negative charge of DNA. To overcome this obstacle, coating the spores with a positively charged intermediate reagent could provide a suitable substrate for DNA adsorption. Nonetheless, the pretreatment of spores employing reagents currently used in plasmid delivery systems, such as spermidine, CaCl2 and CTAB, did not allow efficient DNA binding to spores (Table 1). However, pretreatment of spores with DODAB vesicle fragments promoted a higher adsorption efficiency of plasmid DNA even without extended incubation periods. The adsorption process is faster and simpler than previ-
ously described procedures for binding supercoiled plasmid DNA on other particulate substrates (Svarovsky et al., 2008). Treatment of spores with DODAB also allowed the binding of oligonucleotides at the same conditions established for double stranded DNA, which is not observed with conventional gold particles (Svarovsky et al., 2008). This property could be particularly useful for immunizations with the immunomodulator molecule CpG. Indeed, Rozenfeld and colleagues (Rozenfeld et al., 2012) reported that the administration of DODAB bilayer fragments combined with CpG oligonucleotides induced enhanced immunological effects after parenteral administration with protein antigens.

Regarding future biotechnology applications, we evaluated the performance of the spore-DODAB-DNA system as a particulate carrier for gene gun delivery. Our results demonstrated that inactivated spores coated with a plasmid encoding the luc2 reporter gene could transfect eukaryotic cells, emitting similar luminescence levels as those achieved with gold particles, but in clearly reduced areas of transfected cells (Fig. 3). This could be related to the fact that the spores are much lighter than the gold particles, causing a greater loss of material during the gun shot. Besides, DNA-coated spores presented an elevated PDI (reaching 1.0 for killed spores), indicating that the sample has a very broad size distribution and may contain aggregates derived from the agglomeration of the neutral spore-DNA complexes that could influence the transfection efficiency. Based on this result, the next transfection experiments were performed using twice the amount of DNA for the spores cartridges. Interestingly, live B. subtilis spores did not promote efficient transfection of cells or mouse skin. The germination of the spores in to the cells (Duc et al., 2004; Leser et al., 2008; Ceragioli et al., 2009) causes disassembling of the spore-DNA complexes and protease

production during the bacterial cell replication (Prestidge et al., 1971; Nakayama et al., 1977; Takamatsu et al., 2000), which could lead to the degradation of the recombinant protein.

In the vaccinology context, B. subtilis spores demonstrated immunostimulatory effects when co-administered with protein antigens (Song et al., 2012; Barnes et al., 2007; Cerovic et al., 2009; Liang et al., 2013). The adjuvant effects of spores have been observed both after administration via mucosal (Huang et al., 2010) and parenteral routes (Aps et al., 2015; Souza et al., 2014). In addition to these observations, our results demonstrated that coating B. subtilis spores with plasmid DNA enhanced the cellular and antibody responses induced by a DNA vaccine after intradermal administration through a gene gun device. These results clearly expand the applications of B. subtilis spores as a platform for delivery and enhancement of immune responses induced by subunit vaccines, either as purified recombinant proteins or as DNA vaccines.

Gene gun administration has been widely used for the administration of DNA vaccines (Stezt and Tüting, 2013; Bryan et al., 2013; Bergmann-Leitner and Leitner, 2013). It allows a drastic reduction in the vaccine amount, promoting similar immune responses compared to intramuscular administration, which requires around 100-fold more immunogen. Although this technique has been successfully tested in both animals and humans, the high cost of the gold microparticles makes this vaccination strategy unsuitable for large scale immunization programs. For the administration of one thousand doses (500 mg of gold particles) the cost can be up to US$ 400. Meanwhile, the results presented herein demonstrate that the use of inactivated bacterial spores can drastically reduce the costs of gene gun immunization (approximately 25 cents for 1000 doses,

Fig. 5. Antigen specific cellular and humoral responses elicited in mice immunized with a DNA vaccine by the gene gun delivery. Mice were immunized twice (14 days of interval) with 2 μg or 4 μg per dose of pGDE7 h in the abdominal region using gold (Gold-vac) or killed spore (KS-vac) particles as biolistic carriers. The control group corresponds to non-vaccinated mice and the KS group to mice immunized with non-coated spores. Individual sera were collected 14 days after the first or second immunization for peripheral blood mononuclear cell (PBMCs) analysis. (A) Frequencies of IFN-γ-producing CD8+ T cells after in vitro stimulation with the E7-specific peptide (80RAHNRVTG) of PBMCs obtained 14 days after the second dose, stained for CD8 (FITC) and intracellular IFN-γ (PE) and monitored by flow cytometry. (B) Specific anti-gD IgG titers determined by ELISA. All data are shown as the mean ± SD based on two independent experiments (n = 8–10). Statistical significance: (*) p < 0.05, (ns) non-significant.
based on the costs of the culture medium components). B. subtilis spores can be produced at larger quantities (up to 5 g of dried mass per liter) and at high purity by fairly simple procedures (Tavares et al., 2013). Furthermore, spores of several species of Bacillus are considered safe for human consumption, including B. subtilis (Hong et al., 2008; Istitico et al., 2001) and can frequently be isolated from soil (Aps et al., 2002). Moreover, B. subtilis spores, in contrast to gold particles, are organic and biodegradable once introduced into mammalian tissues. These experimental evidences, thus, add another application of B. subtilis spores in vaccine technologies and shall support further use of the gene gun administration of vaccines.

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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References


