Effects of the liposomal co-encapsulation of antigen and PO-CpG oligonucleotide on immune response in mice

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Abstract

The development of novel vaccines requires the design of new adjuvants able to give long lasting immune responses. Our aim was to obtain cationic liposomes as adjuvants by an industry-suitable method, and evaluate them using bovine serum albumin (BSA) as immunogen and CpG oligonucleotides with phosphodiesther bonds, as immunostimulants. Liposomes (Lip) were prepared with dipalmitoylphosphatidylcholine, cholesterol and stearylamine by Ethanol Injection method. Immune response was assessed by immunization of Balb/c mice with: Lip+BSA Lip+BSA+CpG, CpG+BSA or aluminium hydroxide ($Al(OH)_3$+BSA). Liposomal formulations were able to induce high antibody levels. Lip+BSA+CpG led to higher IgG levels than Lip+BSA ($p<0.05$, Mann-Whitney) and elicited the highest IgG2a levels. All the formulations induced antigen specific cellular proliferation, without significant differences, meanwhile Lip+BSA+CpG produced the highest levels of IFN-γ. These results showed these liposomes are versatile vehicles to potentiate and target the immune system to vaccination, leading to high humoral and cellular immune responses.

Keywords

Vaccines, adjuvant, liposomes, CpG-ODN, humoral response, IFN-γ.

1. Introduction

Vaccines development is considered one of the 20th century scientific goals. Nowadays vaccination is a key for infectious diseases control, preventing 2 to 3 million deaths per year from diphtheria, tetanus, pertussis and measles, as the World Health Organization reported in July 2016 (WHO). An ideal vaccine must be highly efficacious, economical, safe, stable and able to generate a protective immune response against infectious diseases (Perrie, et al., 2008).

The appearance of molecular biology and genetics engineering has enabled the production of new safe vaccines using highly purified recombinant proteins or subunits of pathogens. They are called subunit vaccines and have some advantages over live attenuated and killed inactivated vaccines, such as the elimination of biohazard associated with pathogenic organisms handling and final product virulence. However, their major drawbacks are weak immunogenicity and short-term immune responses, turning the use of adjuvants in the key to enhance and modulate the immunogenicity of the vaccine antigen (Ada, 2005; Nascimento & Leite, 2012). An ideal adjuvant should be stable with a long shelf-life, safe, economical, biodegradable, immunologically inert and able to promote an appropriate humoral and cellular immune response depending on requirements for protection, reducing the amount of antigen or the number of immunizations, even in new-borns, the elderly or immune-compromised persons (Aguilar & Rodriguez, 2007; Garçon et al., 2011; Maisonneuve et al., 2014).

To date, just a few adjuvants are used in human vaccines, and they present several disadvantages. Aluminium adjuvants, including aluminium hydroxide and aluminium phosphate, are the most widely used. Despite its extensive and continuous use, the immune mechanism of action of aluminium remains incompletely understood. Aluminium adjuvants act primarily to increase antibody production and are therefore suitable for vaccines targeting pathogens eliminated primarily by antibody mediated effector mechanisms. Aluminium-adjuvanted vaccines have not been successful in preventing infection due to intracellular pathogens (Marrack, McKee, & Munks, 2009).

Although it was reported that aluminium hydroxide-based adjuvants could improve both Th1 and Th2 cellular responses using the appropriate vaccination route, it is traditionally considered that they preferentially prime Th2-type immune responses (He et al., 2015).

Therefore, this generates the necessity of novel adjuvants for the development of new vaccines capable of inducing protective and long lasting responses.

One of the new alternatives is the use of liposomes as immunological adjuvants. Liposomes are composed of biocompatible phospholipid bilayers and are capable of loading and delivering hydrophilic and hydrophobic molecules, making possible the co-delivery of antigens and immunostimulatory agents and consequently enhancing a protective immune response. Liposomes are able to induce both humoral and cellular immune response to protein and polysaccharide antigens (Gregoriadis, 1990; Sivakumar et al., 2011). The immune response balance depends on the antigen characteristic as well as liposome properties like composition, electric charge, number of layers, and preparation method (Fahmy et al., 2005; Kim et al., 2014). The principal advantages of liposomes as adjuvants are: (i) the ability to mimic pathogens by transporting large quantities of antigens to antigen presenting cells (APCs), (ii) the possibility of co-delivery of antigen and immunostimulatory agents to APCs, (iii) the capacity to modify their physical and chemical properties to obtain a proper immune response, and (iv) low toxicity and biodegradability (Leserman, 2004).

DNA containing unmethylated CpG dinucleotides (CpG-DNA) are prevalent in bacterial and many viral DNAs, but are heavily suppressed and methylated in vertebrate genomes. CpG motifs promote the induction of Th1 and pro-inflammatory cytokines and support the maturation/activation of professional antigen presenting cells, an effect that underlies their use as immune adjuvants (Gursel et al., 2002; Hartmann et al., 2000; Klinman et al., 2004). Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs and phosphorothioate-modified (PS-ODN) which are resistant to nuclease activity have been used in several clinical trials as adjuvant and in therapeutics for allergic and infectious diseases (Ballas et al., 2001; Krieg, 2006). However, many studies have reported that PS-ODN-treated mice could present side effects, such as transient splenomegaly, lymphoid follicle destruction, arthritis, and PS-ODN-specific IgM production (Shargh et al., 2012). Consequently, the use of phosphodiester bond CpG-DNA (PO-CpG) as a natural counterpart of PS-ODN could represent an alternative to enhance an immune response without harsh side effects.

It has been demonstrated that adding CpG ODNs to conventional protein antigens improves the production of antigen-specific antibodies. However, CpG-ODN's half-life is less than 60 minutes,

even using PS-ODN, due to rapid elimination by serum adsorption; therefore, it is necessary to prolong their bioavailability (Gursel et al., 2001; Kim et al., 2011). The use of liposomes to co-encapsulate the CpG-ODN and a protein antigen is a valid method to raise the oligonucleotide half-life and allow an APC to capture both molecules, which could increase the chances of enhancing a specific immune response to the antigen (Krishnamachari & Salem, 2009; Song et al., 2014). Some authors have reported that the use of CpG-ODN combined with liposomes could increase a Th1 immune response to a protein antigen (Bal et al., 2011; Hartmann et al., 1999; Suzuki et al., 2004; Warren et al., 2000).

Given the liposomes ability as vehicle and the immunostimulant effect of PO-CpG oligonucleotides, this study was aimed to evaluate the immune response elicited by their combination in a murine model, using bovine serum albumin (BSA) as immunogen. Moreover, we characterized the cationic liposomes obtained by ethanol injection method.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol) and stearylamine (SA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The PO CpG used in this study was a 20-mer 5'-tccatgacgttcctgacgtt-3' (Badiee et al., 2008; Shargh et al., 2012), synthesized by Invitrogen (Waltham, MA, USA) with phosphodiester backbones.

Unless specified, chemicals were of analytic grade and purchased from Sigma-Aldrich (St. Louis, MO, USA), and electrophoretic reagents were from Bio-Rad (Hercules, CA, USA).

2.2. Animals

Six-8 weeks old, female BALB/c mice (n=6/group), were provided by the Centro de Medicina Comparada, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral (Esperanza,

Santa Fe, Argentina). Animals were kept in the animal holding facility of the Laboratorio de Inmunología Básica - Facultad de Bioquímica y Ciencias Biológicas-Universidad Nacional del Litoral (Santa Fe, SF, Argentina), under controlled ambient conditions. They were provided food and water *ad libitum*. All experimental procedures were approved by the institutional ethical committee and were performed according to the Guide for Care and Use of Laboratory Animals recommendations (ILAR, 2010).

2.3. Liposome preparation

Positively charged liposomes were prepared using DPPC:Chol:SA, in 7:2:2 mol/mol ratio and 4mM as final lipid concentration by the ethanol injection method (Wagner et al., 2006). Briefly, the lipids were dissolved in an ethanol/isopropanol mixture (1:1 v/v). The aqueous phase consisted of 50mM acetate buffer (AcB, pH=4.3) containing Bovine Serum Albumin (BSA) as immunogen. Three different concentrations of BSA were assessed: 23, 100, or 200 µg/mL. While the aqueous phase was kept in continuous stirring, the organic phase was incorporated by injection in a 1:9 (v/v) proportion.

2.4. Liposomes with CpG oligonucleotides preparation

Liposomes were prepared as previously described with 8mM final lipid concentration. CpG oligonucleotides were dissolved in AcB 50mM at 15 µM and added to an equal volume of preformed liposomes (Commander et al., 2010), resulting the following formulation: lipids 4mM, BSA 100µg/mL, CpG 7.5µM.

2.5. Liposomes characterization

The average liposome diameters and the size distribution were determined by dynamic light scattering (DLS) (Yang et al., 2012). A light-scattering photometer by Brookhaven Instruments Inc. was employed, fitted with a vertically polarized He-Ne laser at 632.8 nm, and a digital correlator (Model BI-2000 AT). The measurements were carried out at 30°C and a detection angle

of 90°. Each measurement took between 100 s and 200 s, and the particle concentration was adjusted to produce a counting rate of around 200 counts/s. An average diameter (represented by D_{DLS}) can be calculated through the cumulants method (Koppel, 1972) from the measured autocorrelation function and the knowledge of the absolute temperature, the medium viscosity and the real refractive index of the medium. The liposomes size distribution (LSD) based on the intensity of scattered light was estimated from the autocorrelation functions using proprietary software, which requires solving a problem of numerical inversion, and a method of standard regularization (Gugliotta et al., 2010).

The amount of BSA encapsulated in prepared liposomes containing a total of 100µg/mL of BSA (Lip+BSA) was assessed by SDS-Page, after separation of free protein by Vivaspin® 500 centrifugal concentrators (0.2µm) (Sartorius Stedim Biotech S.A., Concord, CA, USA) (Shargh et al., 2012).A 100 µg/mL BSA solution was subjected to the same treatment. Electrophoresis was performed in a running gel of 15%, w/v acrylamide and a stacking gel of 4.8%, w/v acrylamide. Electrophoresis was carried out at 200 V constant voltages for 60 min in 25 mM Tris, 25 mM glycine, 0.1 % SDS, pH 8.3 buffer. After electrophoresis the gel was stained with Coomassie Blue R-250, 0.5% w/v, for protein detection (Laemmli, 1970). The quantitation of BSA was performed by image analysis of the stained gel, using ImageJ programme by comparison with a calibration curve (Abràmoff et al., 2004).

The liposomal formulation containing the CpG oligonucleotide (Lip+BSA+CpG) was subjected to agarose gel electrophoresis to test for CpG loading efficiency. A Lip+BSA+CpG fraction was mixed with loading buffer (30% v/v glycerol, 0.25% w/v Bromophenol Blue) and analyzed by electrophoresis on ethidium bromide-stained 2% agarose gel using Tris-acetate-EDTA (TAE) 1X as electrophoresis buffer. Electrophoresis was carried out at 100 V constant voltages for 20 min. After electrophoresis the gel was visualized using an UV transiluminator (Fotodyne Incorporated, New Berlin, WI, USA) (Balbino et al., 2012; Barichello et al., 2010).

2.6. Murine immunization

Different groups of mice were inoculated 2 times at 3 weeks interval with one of the following formulations, through intraperitoneal (i.p.) or subcutaneous (s.c.) injections: $Lip_{i.p.}$ (n=4),

Lip+$CpG_{i.p.}$ (n=4), $Al(OH)_3$+$BSA_{i.p.}$ (n=6), $Al(OH)_3$+$BSA_{s.c.}$ (n=6), CpG+$BSA_{i.p.}$ (n=3), CpG+$BSA_{s.c.}$ (n=3), Lip+$BSA_{i.p.}$ (n=6), Lip+$BSA_{s.c.}$ (n=6), Lip+BSA+$CpG_{i.p.}$ (n=6) and Lip+BSA+$CpG_{s.c.}$ (n=6). All doses were prepared in a final volume of 100µL; BSA and CpG content were 10µg and 0.75 nmol, respectively. Animals were tail bled one day before and one week after each inoculation. After centrifugation of the blood at 2000 X g for 10 minutes (min), the serum obtained was stored at -20 °C until further analysis.

Mice were bled by cardiac puncture, sacrificed 2 weeks after the last booster injection and their spleens were aseptically removed. Mononuclear cells were isolated by Phycoll-Hypaque (PhycollPaque Plus, GE, Uppsala, Sweden) density centrifugation method from spleen cell suspension, washed with PBS and immediately used for lymphoproliferation assays.

2.7. ELISA

The antibody responses (IgG, IgG1 and IgG2a) to BSA were determined by an indirect ELISA. Ninety six-well flat bottom plates (Greiner Bio-One, Frickenhausen, Germany)were coated overnight with 1.0 µg/well of BSA 100 mM in sodium carbonate buffer (pH 9.6), washed and blocked with 5% (w/v) skimmed milk powder dissolved in PBS at pH 7.4. Plates were washed again with PBS Tween 20 0.05% and sera were tested at appropriate dilutions. Preimmune sera were used as a control. Binding of the antibody was evaluated by incubation with horseradish peroxidase conjugate anti-mouse IgG (Jackson, Baltimore, MD, USA), anti-mouse IgG1 (Santa Cruz Biotechnology, Inc. Santa Cruz, La Jolla, CA, USA) or anti-mouse IgG2a (Abcam Inc., Cambridge, MA, USA), followed by incubation with hydrogen peroxide and tetramethylbenzidine (Zymed, San Francisco, CA, USA), the colour reaction was stopped by adding 2 N H_2SO_4 to the wells. Optical density was measured at 450 nm using a microplate reader (EmaxMicroplate Reader, Molecular Devices, Sunnyvale, CA, USA).

2.8. Cell proliferation assay

Mononuclear cells were suspended in complete RPMI 1640 (Gibco®, Gaithersburg, MD, USA) culture medium and $2x10^5$ cells/well were cultured by triplicate in 96-well flat-bottom plates

(Greiner Bio-One, Frickenhausen, Germany). The cells were stimulated *in vitro* with either phytohaemagglutinin according to manufacturer's instructions (Gibco®), or BSA (5 µg/well), and incubated at 37°C in 5% CO_2 for 72 h. Cell proliferation was evaluated by Bromodeoxyuridine (BrdU) incorporation assay, according to the manufacturer's instructions (Cell Proliferation Biotrack ELISA, GE, Uppsala, Sweden). Results were expressed as Proliferation Index (PI) = [OD(stimulated) – OD(blank)]/[OD(unstimulated) – OD(blank)]. A PI ≥ 2 was considered representative of specific proliferation.

2.9. Cytokine measurement

Mononuclear spleen cells were cultured as previously described (Soutullo et al., 2005). Briefly, the cells were washed and resuspended in complete RPMI and seeded at $2x10^6$cells/well in 24-well flat-bottom plates (Greiner Bio-One, Frickenhausen, Germany). The cells were stimulated *in vitro* with BSA (50 µg/well) or cultured in medium alone, as described. The culture supernatants were collected after 72 h and used for IFN-γ and IL-4 determination by ELISA method, according to the manufacturer's instructions (BD OptEIA™, San Diego, CA, USA). The concentrations, expressed as pg/mL, were determined by reference to the corresponding standard curves.

2.10. Statistical analysis

Results are reported as mean ± Standard Error of the Mean (S.E.M.) for each group. Statistical differences between groups were analyzed by Mann-Whitney test or Kruskal-Wallis test, followed by *post hoc* comparisons, when appropriate. P values ≤ 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Liposome characterization.

Cationic liposomes were obtained by ethanol injection. Average diameters determined by DLS were 234.7nm, 203.7nm, 192.7nm y 125.5nm, for BSA 0µg/mL, 23µg/mL, 100µg/mL, and 200µg/mL, respectively. Liposomes were stable in the DLS measurement conditions, i.e., diluted samples and to temperature and time of measurements. The reproducibility of the DLS average diameter was around ±2%. As observed in Fig.1, all formulations showed a similar size distribution; however, the mean value decreased as the antigen concentration increased, demonstrating an inverse relationship between the diameter and the concentration of protein antigen ($p<0.05$, Spearman).

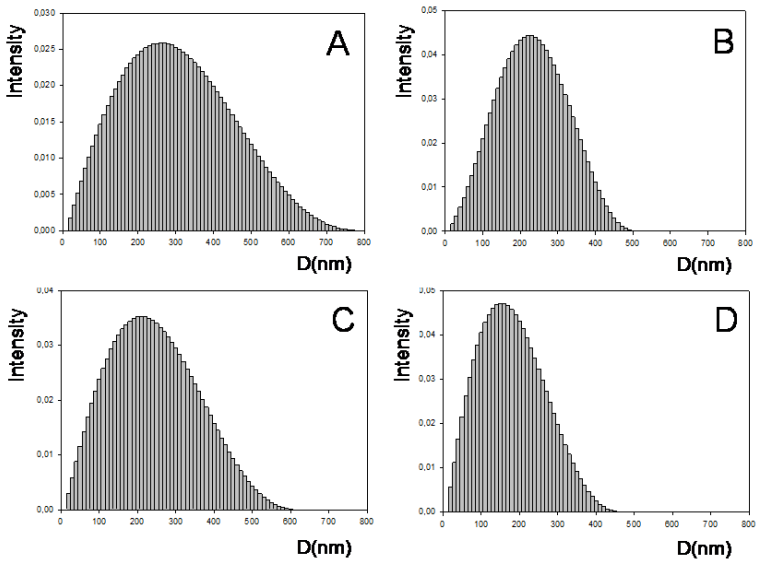

Fig. 1. Liposomes size distribution based on the scattered light intensity (h(D)) and diameter (D(nm)) determined by DLS technique for different protein concentrations. D_{DLS}: mean diameter. **A-**BSA 0µg/mL, D_{DLS}=234.7 nm; **B**- BSA 23µg/mL, D_{DLS}=203.7 nm; **C**- BSA 100 µg/mL, D_{DLS}=192.7 nm; **D**- BSA 200 µg/mL, D_{DLS}=125.5 nm.

Given that the formulation containing BSA 100µg/mL had an appropriate particle size and allowed the injection of 10 µg/dose in an adequate volume, this antigen concentration was chosen for further characterization, supplementation with CpG, and immunization studies.

The BSA entrapment in Lip+BSA was assessed by ultrafiltration and further SDS-PAGE analysis of soluble BSA (Fig. 2-A). Thirty percent of the protein was retained in the liposomal formulation, demonstrating that the antigen was efficiently incorporated into the vesicles.

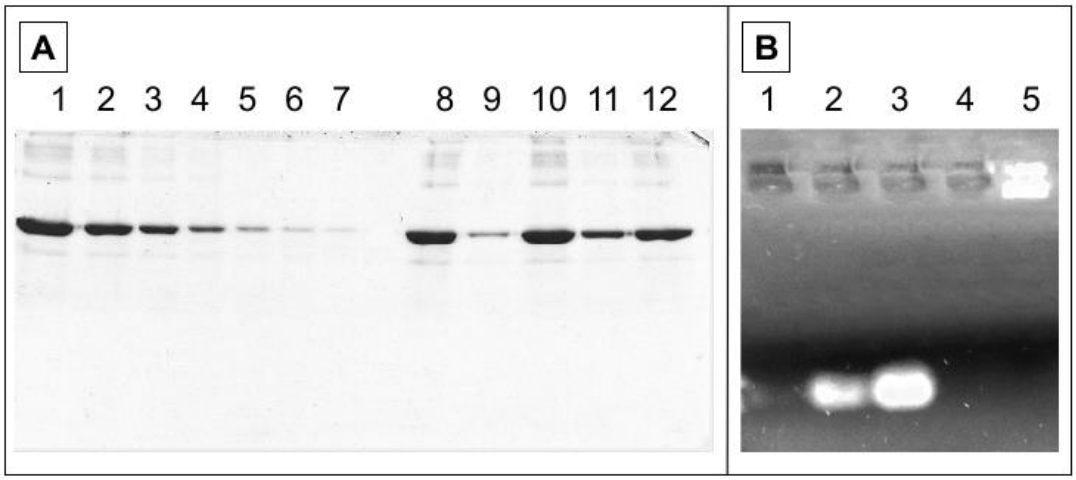

Fig. 2. **A-** SDS-PAGE analysis of BSA incorporation into liposomes. Lane 1 to 7, BSA calibration curve (100.00; 50.00; 25.00; 12.50; 7.25; 3.62; 1.81 µg/ml); lane 8: Lip+BSA before centrifugation, lane 9: BSA retained in the filtration unit; lane 10: filtered BSA; lane 11: retained BSA in the filtration unit after Lip+BSA centrifugation; lane 12: filtered BSA after Lip+BSA centrifugation. **B-** Agarose analysis of CpG incorporated into the liposomes. Lane 1 to 3, CpG solution (0.75; 3.75; 7.50 nmol/ml); lane 4: Lip+BSA; lane 5: Lip+BSA+CpG.

The Lip+BSA suspension stability at 4ºC was assessed weekly by visual inspection, and after 9 months mean liposome diameter was determined again by DLS. The formulation was stable throughout the study period and particle size did not change at the second evaluation (D_{DLS}=196.1 nm; $p>0.05$).

CpG containing liposomes were obtained as described in 2.1 section. Although the resulting suspension was stable during at least 1 hour by visual inspection, turbidity was increased in comparison with control samples, and DLS could not be performed due to the lack of Brownian motion of particles.

As shown in Fig. 2-B, no free DNA was detectable in the presence of liposomes suggesting that most of oligonucleotides were complexed with liposomes.

3.2. Immunization studies

3.2.1. Humoral immune response assessment

In order to assess the formulations adjuvanticity and the influence of the inoculation route (subcutaneous, s.c., or intraperitoneal, i.p.) upon the immune response, Balb/c mice were immunized as described above. $Al(OH)_3$ was used as a control adjuvant. No production of anti-BSA antibodies were detected in preimmune animal serum (data not shown).

After the first dose, Lip+BSA+CpG induced the highest antibody levels by s.c. route ($p<0.05$). No differences between routes were observed for any of the formulations except for $Al(OH)_3$+BSA, which showed significantly higher levels of antibodies by the i.p. route (Fig.3)

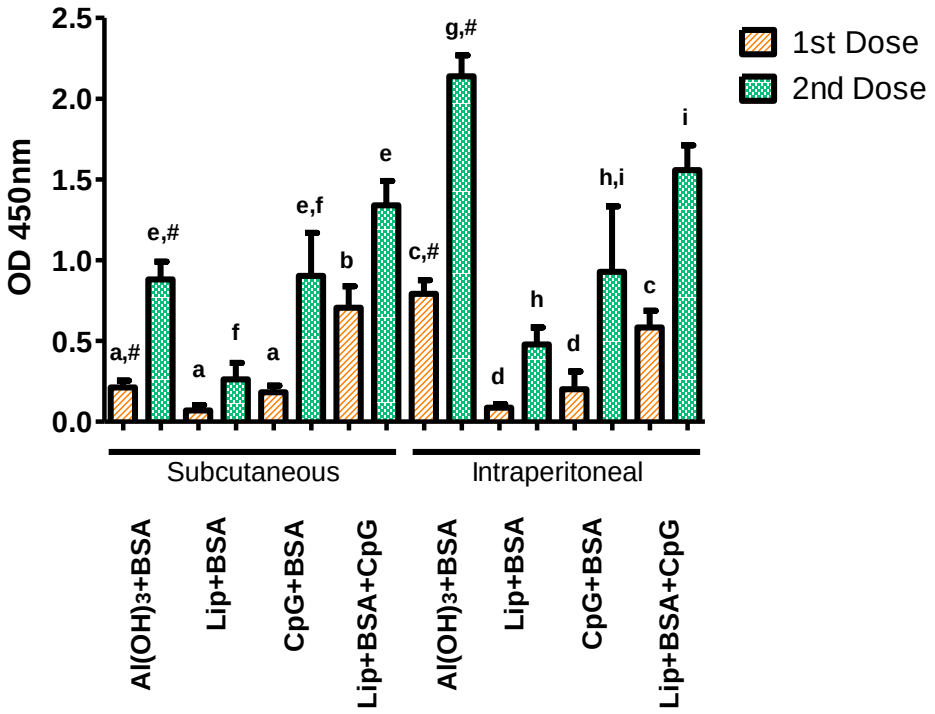

Fig.3. IgG total levels after the first and second doses, assessed by ELISA. Different letters indicate significant differences among formulations for the same route and doses; #: significant differences between routes for the same formulation and doses ($P<0.05$).

After the second dose all the formulations, but Lip+BSA, were able to increase the antibody levels ($p<0.05$). By the s.c. route, Lip+BSA+CpG induced similar IgG levels to those obtained with the control adjuvant (Al(OH)3+BSA), while this adjuvant gave significantly higher antibody levels than liposome based formulations, only by the i.p. route. No production of anti-BSA antibodies were detected in control groups inoculated with BSA without adjuvant, Lip, or Lip+CpG (data not shown).

IgG levels after the second dose were quantitated by titration. Fig. 4 represents the title of each serum expressed as the last dilution exceeding the OD average of negative controls + 2 SD.

IgG titles induced by Lip+BSA+CpG were similar to those induced by $Al(OH)_3$+BSA, by both routes, and significantly higher than the rest of the formulations. Lip+BSA+CpG was the only formulation that exhibited a significant difference between immunization routes, giving higher IgG levels by the s.c. compared to the i.p. route ($p<0.05$). CpG ODN without liposomes was able to potentiate the antibody production against BSA at similar levels than liposomes without CpG, by both routes (Fig. 4).

IgG1 and IgG2a subclasses were analyzed after the second dose. Regarding IgG1, both $Al(OH)_3$+BSA and Lip+BSA+CpG helped to reach the highest levels of this subclass, by both routes of administration. Lip+BSA and Lip+BSA+CpG induced similar levels of IgG1. Furthermore, when analyzing IgG2a, Lip+BSA+CpG induced significantly higher antibodies levels than any other formulation by both routes.

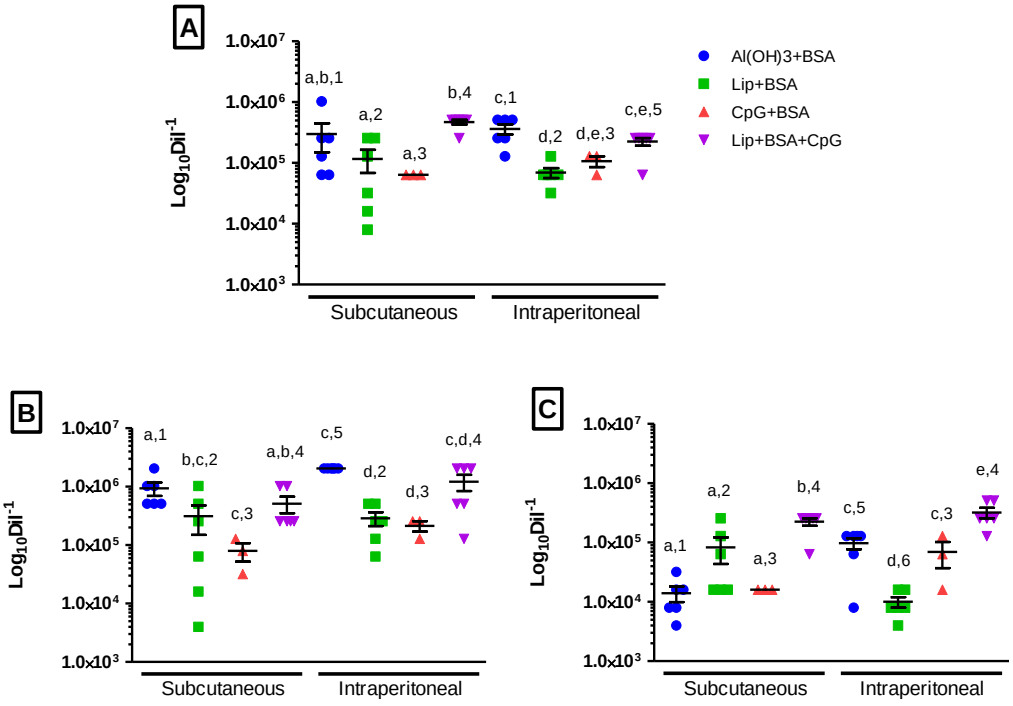

Fig.4. Antibody titles after the second dose, assessed by ELISA. **A-**Total IgG, **B-** IgG1, **C-**IgG2a. Different letters indicate significant differences among formulations for the same route; different numbers indicate significant differences between routes for the same formulation ($P<0.05$).

3.2.2. Cellular immune response assessment

Cellular immune response was evaluated by proliferation assays with mononuclear cells obtained from spleens of immunized mice. Mice from all groups had cells that were able to proliferate in response to the antigen (PI≥2), with the exception of two animals (one from Lip+BSA+CpGs.c. and the other from $Al(OH)_3$+$BSA_{i.p.}$ These mice also exhibited the lowest antibody titles. No differences in PI were observed among groups (data not shown).

3.3.3. Cytokine measurement

IL-4 and IFN-γ levels were determined in the supernatants from cultured cells after 72 hours of stimulation. Cells from mice immunized with Lip+BSA+CpG by both routes produced the highest levels of IFN-γ. This increase was statistically significant for the i.p. route of injection compared with $Al(OH)_3$+$BSA_{i.p.}$ ($p<0.05$). IL-4 was slightly produced by the cells from all groups, being significantly higher for the group Lip+BSA+$CpG_{i.p.}$ compared with $Al(OH)_3$+$BSA_{i.p.}$ ($p<0.05$) (Fig. 5).

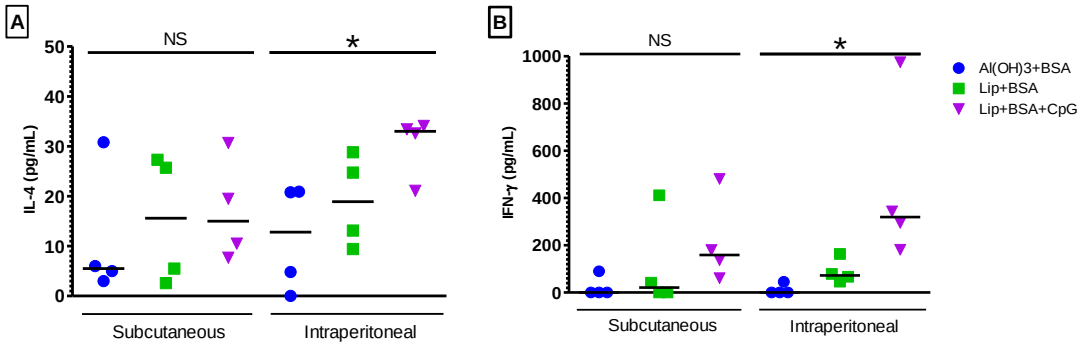

Fig.5. Cellular immune response assessment. **A-** IL-4 and **B-** IFN-γ concentration in culture supernatants after 72 h of stimulation assessed by ELISA. *$p<0.05$, Lip+BSA+$CpG_{i.p.}$ vs. $Al(OH)_3$+$BSA_{i.p.}$ NS: not significant.

4. Discussion

Subunit vaccine development has been an area of great expansion during the last decade, and new adjuvants are needed to accompany this progress. Liposome based formulations emerge as

a promising tool giving their properties, particularly their plasticity. Here we describe the design, production, and characterization of cationic liposomes to be used as vaccine adjuvants.

These liposomes are constituted by DPPC, which is the main component of the lipid bilayer, cholesterol, which gives fluidity and contributes to the impermeability, and stearylamine, which provides the positive charge to the vesicles. The method of preparation described here, the ethanolic injection, allowed the generation of particles with a relatively narrow distribution of sizes, and mean diameters ranging from 125.5 to 234.7 nm. As previously reported, these diameters are compatible with oligolamellar vesicles, but further analysis are required to confirm it (Laouini et al., 2012). The classic method of lipid film rehydration was also tested (data not shown) but this procedure led to the generation of a more heterogeneous population of particles, with sizes up to 5µm, and a weak stability in suspension. Moreover, the method is hard to be scaled-up, which represents a limitation for mass production of liposomal formulations. Particles obtained in this work resulted in a stable suspension, for up to 9 months. This avoids the need of product lyophilization as preservation method, at least during this period of time.

Thirty percent of the antigen was incorporated into the vesicles, in concordance with incorporation rates previously described for liposomes obtained in similar conditions (Wang & Huang, 2003). This suggests that liposomes actually play a role as an antigen vehicle in the vaccine formulation (M. Gursel et al., 2002; Hartmann et al., 2000; Klinman et al., 2004).

Oligonucleotides with non-methylated CpG sequences are TLR9 agonists; therefore, they could be used as immunostimulant molecules (Gursel et al., 2002; Hartmann et al., 2000; Klinman et al., 2004). ODN 1826 is a mouse specific, B type ODN, known as Th1 response inducer (Ballas et al., 2001). This ODN is commercially available as a sequence containing phosphorothioate bonds, which renders the internucleotide linkage resistant to nuclease digestion. However, the cost of production of this type of ODN is higher than those with phosphodiester bonds. Moreover, the long-term use of modified links has been associated with side effects in mice as arthritis, transient splenomegaly, and destruction of follicular lymphocytes. In primates, acute inflammation with hemodynamic changes was observed (Shargh et al., 2012). In this work, we evaluated the use of the ODN sequence equivalent to 1826 ODN, but with phosphodiester bonds. Incorporation of ODN into liposomal vesicles would protect it from nuclease degradation. Moreover, phosphodiester CpG could stimulate a TLR-9 independent pathway of cell activation, driving up-

regulation of CD40 and CD69, when it is complexed with a lipid vehicle (Yasuda et al., 2005). ODN was added either in the aqueous phase or in the liposome suspension, but the former method led to liposome aggregation and immediate precipitation (data not shown), while the latter gave a more stable suspension.

Although the addition of ODN increased the turbidity of the liposome suspension, it remained stable for at least one hour. The turbidity increase suggests the formation of aggregates, which can be due to the interaction between the negative charges of a molecule of oligonucleotide with the positive charges of different liposomes. These aggregates were associated with lower stability than liposomes alone, then this formulation should be used during the first hour of preparation. ODN was almost fully incorporated into the liposomes, suggesting that the antigen would get into the APCs in the same particle than the immunostimulant molecule, as it is recommended for full activity of the adjuvant (Krishnamachari & Salem, 2009; Song et al., 2014).

Liposomes adjuvanticity was tested by mice immunization, through different routes of inoculation. Both liposomes and ODN alone were capable of boosting the humoral response against BSA, suggesting their role as vehicle and immunostimulant, respectively. However, their combination led to higher levels of antibodies. The humoral response induced by the liposomal formulations was independent of the immunization route.

Regarding IgG subtypes, $Al(OH)_3$ induced the highest levels of IgG1. Adding of CpG ODN to liposomes had no effect on the levels of IgG1. On the other hand, liposomes with CpG ODN induced the highest production of IgG2a. Splenocytes of mice that received this formulation produced the highest levels of IFN-γ after stimulation. Altogether, these results suggest that immune response induced by this adjuvant is associated with a Th1 profile. This is in concordance with previously reported results (Chu et al., 1997). Although CpG ODN without liposomes were able to induce an immune response, the association with liposomes increased the IgG and IgG2a titres, suggesting that incorporation to particles gave protection against nucleases and/or allowed a higher availability of the complex antigen-immunostimulant to the APCs.

In summary, cationic liposomes were a suitable vehicle for the transport of both a protein antigen and an immunostimulant molecule, and its adjuvanticity was independent of the route of inoculation. They are versatile particles that may be obtained by a relatively simple and economic method. Moreover, we demonstrated that the combination of a natural and low-cost PO-CpG with

liposomes could increase the CpG-ODN immunostimulant effect and diversify the immune response stimulated by liposomal adjuvants.

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