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Effect of cationic liposomes/DNA charge ratio on gene expression and antibody response of a candidate DNA vaccine against Maedi Visna virus

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ABSTRACT

Maedi Visna virus (MVV) is an ovine lentivirus with high prevalence all over the world. Since conventional vaccines had failed in protecting animals against the infection, the development of a DNA vaccine can be an alternative. The candidate vaccine was constructed by cloning the sequence encoding MVV p25 protein and was tested both *in vitro* and *in vivo* experiments associated with cationic liposomes. The lipoplexes (plasmid DNA–liposome complexes) with charge ratios ranging from 0 to 18 were prepared in physiological saline solution and characterized at a physical-chemistry level. Agarose gel electrophoresis was used as a first approach to evaluate qualitatively the amount of unbounded DNA by the liposomes. Dynamic light scattering measurements revealed that under the studied conditions lipoplexes with theoretical charge ratios (+/−) from 3 to 6 are unstable and prone to aggregation displaying sizes higher than 1 μm. At lower and higher charge ratios lipoplex size range from 200 to 500 nm. Using a Foster Resonance Energy Transfer methodology previously reported by us, complexation efficiency of the same complexes was related to *in vitro* and *in vivo* results.

Higher transfection efficiencies were obtained *in vitro* with lipoplexes with charge ratio (+/−) = 10, where 97% of the DNA were protected by the liposomes. However, the subcutaneous immunization of mice induced higher antibody titers with lipoplexes at charge ratio (+/−) = 1, in which only 23% DNA is protected by the liposomes. Moreover, use of cationic liposomes has shown an increased antibody response when compared with a naked DNA immunization.

1. Introduction

Originally isolated in Iceland, Maedi Visna virus (MVV) is a retrovirus of the *Lentivirus* genus that causes a chronic, slowly progressive disease of sheep characterized by a relatively long asymptomatic period that leads to pneumonitis (Maedi) and paralysis (Visna) and ends with the animal death (Sigurdsson, 1954). The 9.2 kb genome of MVV has three major genes, *gag*, *pol*, and *env*, which encode the virus structural proteins. The core proteins p16, p25, and p14 are encoded by the *gag* gene, while *pol* encodes the virus polymerase, and *env* encodes the envelope glycoproteins gp41 (TM) and gp135 (SU) (Henriques et al., 2007; Power et al., 1995; Sonigo et al., 1985).

Maedi Visna virus exists in most of Europe, Asia, Africa and North America countries. This makes it necessary to adopt measures to control MVV infection. Since conventional vaccines have failed in inducing an effective immune response (Cutlip et al., 1987; Larsen et al., 1982; Petursson et al., 2005), the development of a DNA vaccine against MVV can be an alternative (Henriques et al., 2007). In a DNA vaccine the antigen gene is cloned into a plasmid and is expressed within the animal host. Once synthesized, the antigen is presented to the immune system, generating an immune response and an immunological memory that will hopefully protect the animal host from the disease. DNA vaccines can be injected in saline, but enhanced immune response requires alternative delivery methods of DNA administration in order to activate cells of the innate immune system. One of those delivery methods consists on the use of cationic liposomes that not only assist DNA into penetrating the cell, but also prevent its degradation by cellular nucleases (Yang and Huang, 1998). In fact, the use of liposomes in DNA vaccines, requires smaller amounts of plasmid DNA, and has shown enhanced immunity in mice when compared with the immunization with naked plasmid (Gregoriadis et al., 1997). Encouraging results were also obtained in the development of liposome based-DNA vaccine for important pathogens such as HIV (Locher et al., 2004), hepatitis C (Jiao et al., 2004), tuberculosis (D'Souza et al., 2002), malaria (Hartikka et al., 2001) and against antrax spores (Hermanson et al., 2004). The physical-chemical characterization of the cationic lipid/DNA complexes (lipoplexes) before *in vitro* or *in vivo* experiments has been taken into account in order to understand and

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improve the effect of liposomes in DNA vaccination (Perrie et al., 2001). In fact, several methodologies, mainly using fluorescence spectroscopies are routinely used to characterize lipoplexes and predict the behaviour of these complexes *in vivo* and *in vitro*, either in gene therapy or DNA vaccine area (Ausar et al., 2008).

This paper describes the preparation and physical-chemical characterization of a liposome-based vaccine against Maedi Visna virus followed by its transfection efficiency evaluation *in vitro* and its immunogenicity assessment *in vivo*.

2. Materials and methods

2.1. Vaccine construction and production

The candidate DNA vaccine, lacZp25, was constructed using pVAX1lacZ (Invitrogen) as the backbone vector. The sequence encoding p25 protein was amplified by PCR from a strain of MVV isolated in Portugal (P1OLV) (Barros et al., 2004), using primers designed to contain *Nhe*I and *Afl*II restriction sites respectively at the 5′ and 3′ ends of the amplicon. Start codon was inserted with forward primer and reverse primer contains two additional bases, in order to bring MVV and *lac*Z genes into the same ORF and thus codifying the same fusion protein. The primer set was GCGCGCTAGC**ATG**GCCATAGTAAATTTACAAGCAG (*Nhe*I site underlined and start codon in bold) as forward primer and the reverse primer GATACTTAAGCC**CAA**TTGCATTTTAAATCCTTCTG (*Afl*II site underlined and the codon before stop in bold. Once amplified, the fragment was digested with restriction enzymes *Nhe*I and *Afl*II and cloned into pVAX1*lac*Z. The resulting plasmid, named lacZp25 was used to transform *E. coli* DH5α competent cells and transformants obtained were screened by restriction analysis. The nucleotide sequence of the insert was confirmed by automatic sequencing, using *Thermo Sequenase Primer Cycle Sequencing kit* (Amersham Pharmacia Biotech).

Candidate vaccine was produced in *E. coli* DH5α and purified by a process based on hydrophobic interaction chromatography (Diogo et al., 2005; Diogo et al., 2001) and according to regulatory agency recommendations (U.S. Department of Health and Human Services Food and Drug Administration (FDA), 2007). The plasmids were eluted in PBS buffer (0.9% NaCl, 10 mM sodium phosphate, pH 7.2).

2.2. Liposome and lipoplex preparation

Cationic liposomes were prepared at 0.5–4 mM concentration in cationic lipid DOTAP (1,2-Dioleoyl-3-trimethylammonium-propane) (Avanti Polar Lipids). The appropriate amount of lipid was diluted in chloroform solution. The solvent was evaporated under a nitrogen stream to obtain a thin lipid film. Residual solvent was removed under vacuum overnight and the lipid films were hydrated in PBS (pH 7.2). To obtain large unilamellar vesicles (LUV, diameter approximately 100 nm) the hydrated lipid dispersions were extruded 5 times through 0.4 μm and 10 times through 0.1 μm pore diameter polycarbonate filters (Whatman, Clifton, NJ), successively. The liposomes were then stored at 4 °C. The lipoplexes (cationic liposomes-DNA complexes) were obtained by direct and rapid addition of an appropriate amount of the cationic lipid dispersion to the DNA plasmid solution at various charge ratios (DOTAP/DNA between 0 and 18), by adding rapidly equal volumes. The complexes were incubated at room temperature for at least 30 min, before use.

2.3. Lipoplex characterization

Characterization of all plasmid batches was carried out by loading samples (20 μL) in a 0.8% agarose gel, under a constant electric field of 2.0 V/cm with 40 mM Tris-acetate, 1 mM EDTA (TAE) as electrophoresis buffer. Lipoplexes samples, 40 μL, were also analyzed by electrophoresis using the same procedure. All the gels were post-stained in ethidium bromide (0.5 μg/mL) for 30 min and then observed, integrated and photographed with an ultraviolet transillumination equipment (Eagle Eye II, vers.1.1, Stratagene (Cedar Creek, TX)) with a CCD camera system.

Plasmid DNA and lipoplexes size were measured using a Brookhaven Instrument (Brookhaven, NY) with a Multi Angle Sizing Option on the Zeta Plus (BI-MAS) using a 15 mW argon ion laser at 635 nm.

Lipoplex complexation efficiency was determined as described elsewhere (Madeira et al., 2008). In brief, a time-resolved FRET methodology was used to evaluate quantitatively the lipoplex structural changes when prepared in high ionic strength solutions. A theoretical formalism describing the changes in donor ((d)DNA-bound probe) fluorescence in presence of nearby acceptors ((a) Membrane probe molecules), assuming a multilamellar arrangement (Fig. 1) is applied to the fitting of the variation of FRET efficiency as a function of acceptor concentration. For each charge ratio the donor-acceptor interplanar distances (d) and the amount of free DNA are recovered (Madeira et al., 2003).

2.4. In vitro experiments

Chinese Hamster Ovary (CHO) cells were grown in F-12 Nutrient Mixture (Gibco™) supplemented with 10% foetal bovine serum, 100 units of penicillin/mL, 100 μg of streptomycin/mL and 50 μg of gentamicin/mL until confluence. After trypsinization, cells were harvested by centrifugation at 230 g for 10 min and resuspended in 10 mL of culture supplemented medium. Cells were seeded in a 24-well plate with an initial cellular density of 6×10^5 cells/well followed by overnight incubation at 37 °C and 5% CO_2. For transfection, the plasmid lacZp25 and cationic liposomes were mixed at the charge ratios (+/−) 1, 5 and 10. PBS buffer was also added up to 200 μl of final solution and the mixture was incubated at room temperature for 30 min. The cells culture medium was replaced by this mixture and incubated at 37 °C for 2 h. Supplemented culture medium (800 μl) was added to each well and cells were incubated again at 37 °C for 22 h.

Electroporation was performed at 200 V, 40 kHz with 7 ms (×10) with 1 ms intervals using a Gene Pulser II RF Module (Bio-rad) equipment. A cellular density of 5×10^5 cells/200 μl PB-sucrose buffer (272 mM sucrose, 1 mM $MgCl_2$, 7 mM Na_2HPO_4, pH 7.4) with 2 μg of DNA was used.

Protein expression was detected through MVV-β-galactosidase fusion protein, using the β-Gal Staining Set from Roche (Henriques et al., 2007). Cells were incubated at 37 °C for 2 h and detection and counting of blue cells were performed by phase contrast microscopy (Olympus CK2).

2.5. In vivo experiments

2.5.1. Charge ratio (+/−) = 1

In order to perform *in vivo* experiments, lipoplexes were prepared using 20 μg of plasmid DNA with a charge ratio (+/−) = 1. The mixture was incubated at room temperature for 30 min prior to administration.

Seven female BALB/c mice, 6–8 weeks old were immunized subcutaneously with lacZp25/liposome complex and in order to verify the adjuvancy of liposomes, seven mice received 20 μg of lacZp25 without liposomes. Four group controls with seven mice in each were inoculated with (1) plasmid lacZ with liposomes, (2) plasmid lacZ without liposomes, (3) only liposomes, (4) PBS (group with just five mice). The amounts of DNA and DOTAP were the same in all

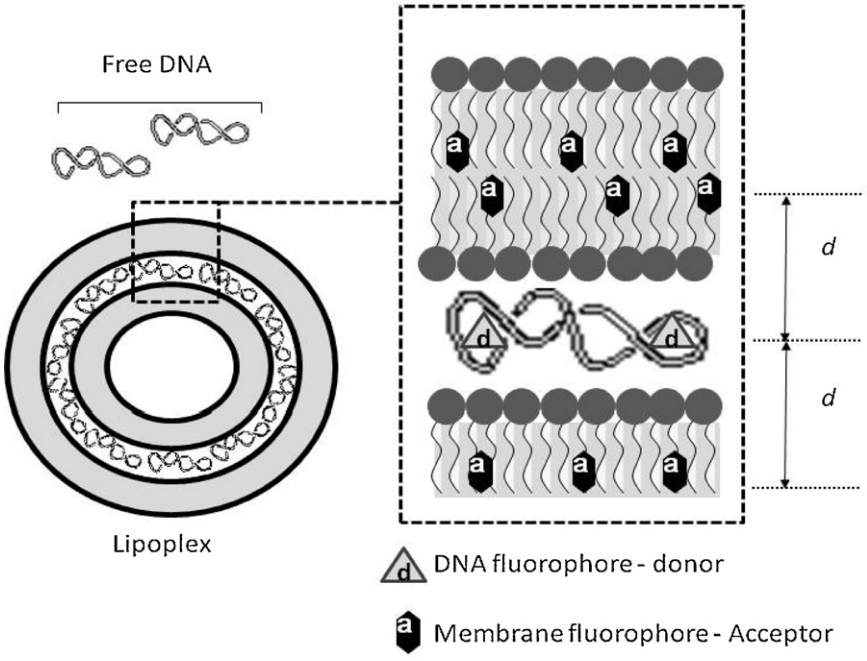

Fig. 1. Lipoplexes multillamelar structure. Zoom section shows a scheme of the used methodology for lipoplex complexation efficiencies quantification using fluorescent probes within DNA and lipid. Acceptor (a) on the lipid (BODIPY-PC) and donor (d) on the DNA (BOBO-1) (Madeira et al., 2003).

experiments. The schedule used comprised a prime administration at day 0 followed by a boost at day 56.

Mice were regularly bled up to 116 days by facial venipuncture and blood was collected to Microtainer™ (Becton Dickinson). The blood was centrifuged at 2000 *g* for 5 min and serum fraction was collected. Pools of sera for each group of mice were prepared by adding the same volume of each mice serum and all samples were stored at −20 °C.

2.5.2. Charge ratio (+/−) = 10

A second experiment was performed using 50 μg of plasmid DNA. DOTAP was added to obtain a charge ratio (+/−) = 10. The solution was also incubated for 30 min at room temperature before inoculation.

Six female BALB/c mice 6–8 weeks old were immunized subcutaneously with lacZp25/Lip complex and to a negative control group of four mice 50 μg of lacZ plasmid without construction were given. Another negative control group of four mice was inoculated with liposomes solution only. Two plasmid administrations were carried out at days 0 and 22. Blood was regularly collected until day 56 and its processing was performed as previously referred.

2.5.3. Antibody response

The antibody response to p25 antigen was measured by ELISA as described elsewhere (Fevereiro et al., 1999; Henriques et al., 2007). In brief, plates were coated with MVV antigen (0.45 μg/μl) and serial dilutions (1:100–1:800) from serum samples were incubated at 37 °C for 1 h. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin and OPD (o-phenylenediamine dihydrochloride) substrate were used to quantify antibodies in sera samples.

The measured absorbance values were plotted as a function of the logarithm of dilution. The obtained values gave rise to a linear representation whose equation was determined. In theory, interception corresponds to the absorbance value of the initial serum solution without dilution. So that, interception value of each line was used as a measurement of antibody response and was represented in another chart as a function of time. A different representation was performed for each group of mice. In order to normalise the different representations, each point was divided by the correspondent value at day 0 (pre-immunization titer). Differences in the antibody titer between mice groups immunized with each plasmid or non-immunized were analyzed performing an ANOVA (General Linear Model, Tukey's comparison test). Analyses were performed with the Minitab statistical package, version 15 (Minitab Inc.).

3. Results

3.1. Lipoplex characterization

The efficiency and extent of cationic liposome-DNA complex formation with different charge ratios were analyzed by agarose gel electrophoresis (Fig. 2). As lipid concentration increases (from left to right), less unbound DNA migrated in the gel. Free DNA is still observed in lipoplexes with (+/−) = 14 (*lane 6*). In our study, according to size measurements shown in Fig. 3, lipoplexes with charge ratios from 3 to 6 seem to be unstable and prone to aggregation, whereas lipoplexes with charge ratio in the 0.5–2 and 8–10 ranges have smaller sizes varying from 200 to 500 nm.

The established fluorescence spectroscopy technique, using a FRET methodology (Madeira et al., 2003, 2008) have allowed us to quantify directly the complexation efficiency of a given lipoplex system and also the "uncovered" or free DNA fraction in lipoplexes, which is susceptible to nuclease degradation (see table in graph in Fig. 3). It was verified that above a charge ratio (+/−) = 5 more than 96% of DNA molecules are surrounded by lipid molecules. In subsequent *in vitro* and *in vivo* studies we used lipoplex with charge ratios (+/−) = 1, 5 and 10.

3.2. In vitro experiments

CHO cells were transfected with lipoplexes in order to confirm protein expression. Transfection was carried out with plasmid lacZp25 and protein expression was assessed indirectly through the β-Gal reporter protein fused to MVV p25 protein, which enzymatic

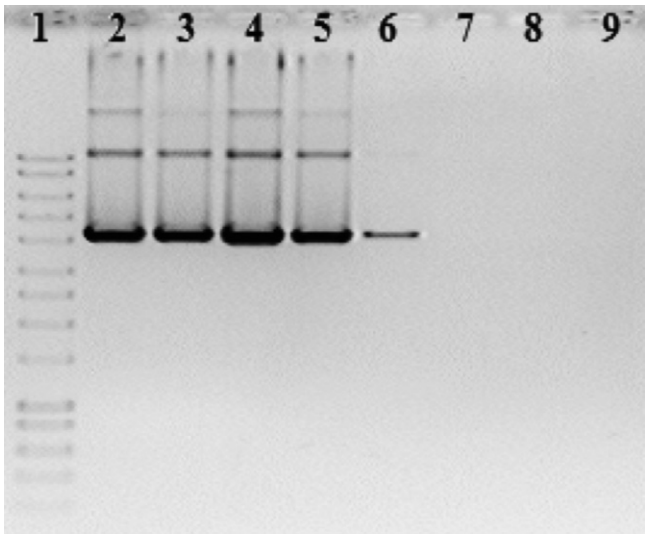

Fig. 2. Agarose gel electrophoresis of lipoplexes DOTAP/lacZp25 prepared with PBS, pH 7.2. HyperLadder I^{TM} marker (*lane 1*); Charge ratios (+/−): 0 (*lane 2*), 1 (*lane 3*), 5 (*lane 4*), 10 (*lane 5*), 14 (*lane 6*), 18 (*lane 7*), 20 (*lane 8*), 22 (*lane 9*) [DNA] = 20 μg/mL.

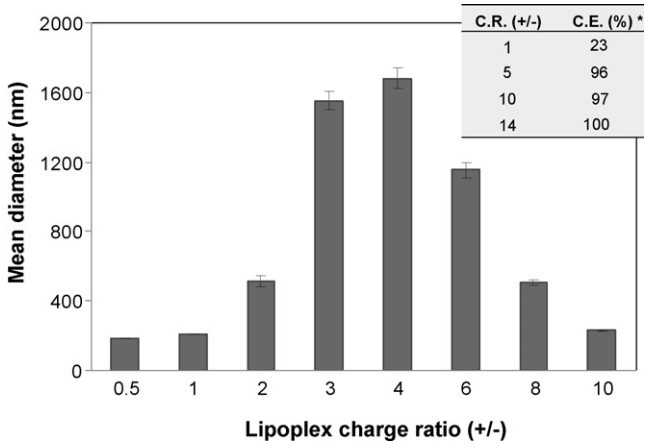

Fig. 3. Mean diameter (nm) of DOTAP/lacZp25 lipoplexes prepared in PBS at several charge ratios (+/−). In the table inserted are shown complexation efficiency (C.E.)* values obtained for several lipoplex charge ratios (C.R.) [DNA] = 20 μg/mL. *(Madeira et al., 2008).

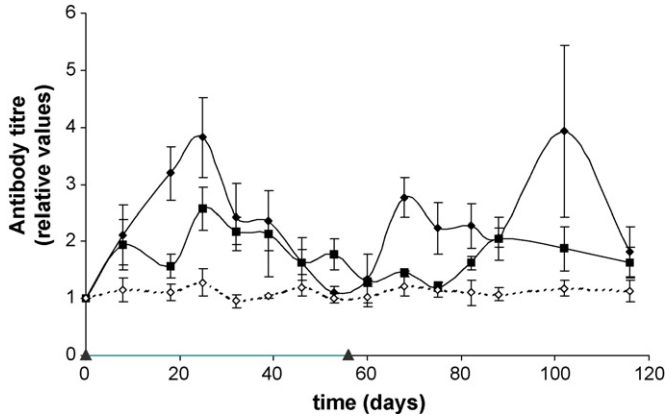

Fig. 5. Antibody response of mice immunized subcutaneously with 20 μg of lacZp25 with (♦) and without liposomes (■) at a ratio charge (+/−) = 1. Control lacZ/Lip (◊) mice antibody response is represented by a broken line. Immunizations were carried out at days 0 and 56 (▲) and each point represents the average of a group of seven mice in three ELISAs. Statistical analyses were performed as described in Section 2. The antibody titers for lacZp25/Lip are significantly higher ($p < 0.001$) than lacZp25. lacZp25 is statistically different from lacZ/Lip and Lip but not from lacZ and NI. All the four negative controls have shown no differences between them (data not shown).

activity originated a blue precipitate. In the three charge ratios tested ((+/−) = 1, 5 and 10) blue stained cells were observed, suggesting that DNA vaccine candidate was correctly constructed and that the prepared lipoplexes could in fact act as delivery method in animal cells transfection (Fig. 4A).

The number of blue stained cells obtained of CHO transfected cells with lacZp25 are represented in Fig. 4B. The highest transfection efficiency (higher number of blue cells) was achieved with charge ratio (+/−) = 10. Transfection efficiency at charge ratio (+/−) = 1 was 1.5-fold higher than at charge ratio (+/−) = 5. Moreover, transfection efficiency of lipoplexes at a charge ratio (+/−) = 10 was compared with electroporation, and it was verified that DOTAP at the referred charge ratio ((+/−) = 10) was more efficient than electroporation for plasmid delivery *in vitro* (Fig. 4C)

3.3. *In vivo experiments*

Fig. 5 shows the antibody response of mice immunized with the different plasmid (20 μg) and liposome combinations. In those experiments, lipoplexes with charge ratio (+/−) = 1 were used. The administration of lacZp25 in a solution containing liposomes elicited a strong antibody response in mice, when compared to that exhibited by mice immunized with lacZp25 in the absence

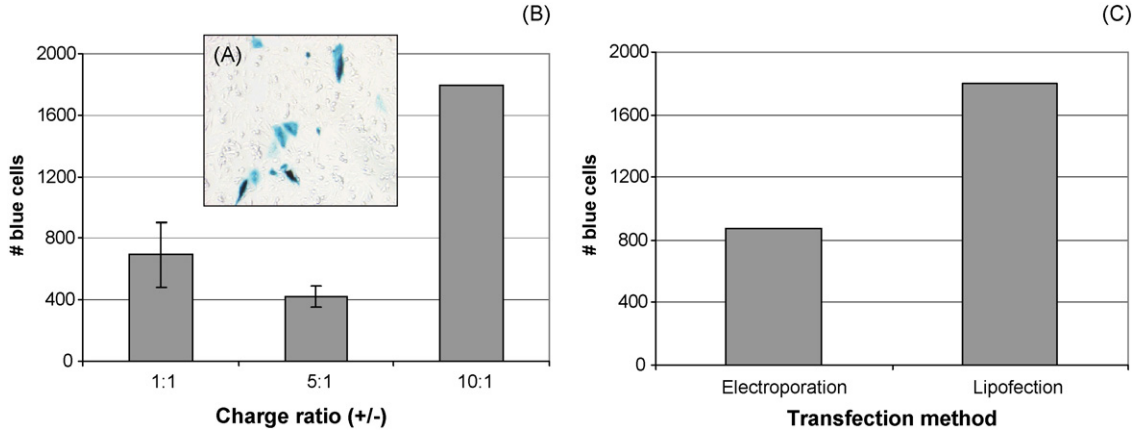

Fig. 4. *In vitro* qualitative (A) and quantitative (B and C) analysis of blue cells obtained after transfection of CHO cells with lacZp25. (B) Lipofection using lipoplexes at ratio charges (+/−) = 1, 5 and 10; (C) Comparison of two different techniques: electroporation and lipofection (charge ratio (+/−) = 10).

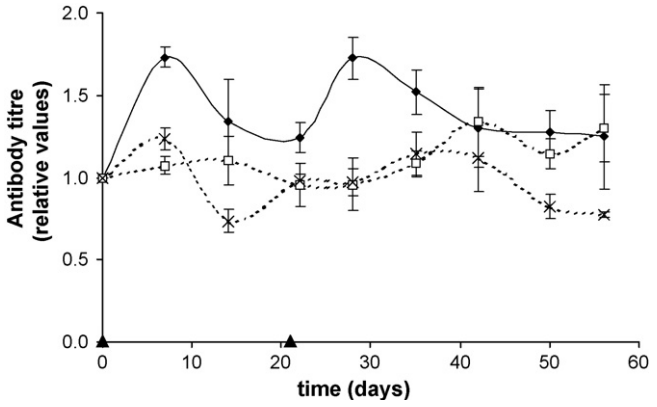

Fig. 6. Antibody response of mice immunized subcutaneously with 50 μg of lacZp25 with liposomes (♦) at a ratio charge (+/−) = 10. Control lacZ (□) and liposomes (×) mice antibody responses are represented by broken lines. Immunizations were carried out at days 0 and 22 (▲) and each point represents the average of a group of six mice for lacZp25/liposomes and of four mice for controls in three ELISAs. Statistical analyses were performed as described in Section 2. The antibody titers for lacZp25/Lip are significantly higher ($p < 0.001$) than lacZp25 and the Lip. lacZ is not statistically different from Lip.

of liposomes. In fact, the antibody titer of mice inoculated with lacZp25/liposomes reached 4-fold the initial value, while the antibody titer of mice immunized with lacZp25 without liposomes only reached 2.5-fold the antibody titer registered before the administration of the candidate DNA vaccine. Moreover, the lacZp25/liposomes boost immunization led to an increase of antibody response that was much higher and faster than that observed by mice immunized with lacZp25 without liposomes. Statistical analyses performed as described in Section 2 have shown that the differences observed between mice immunized with these two formulations were statistically significant ($p < 0.001$). The antibody response of mice immunized with lacZp25 in the absence of liposomes is statistically different from that of mice inoculated with lacZ/Lip and Lip but not of mice inoculated with lacZ and PBS. The antibody titer of all groups of control mice was kept almost constant, exhibiting only a little variation along the experiment, that did not exceeded a maximum of 1.5-fold the initial value. Statistical analyses confirmed that all the four negative controls exhibited no differences between them. Therefore, and for clarity of interpretation, only lacZp25/Lip, lacZp25 and lacZ/Lip groups are represented in Fig. 5.

A similar *in vivo* experiment was performed by the inoculation of six mice with 50 μg of the same plasmid (lacZp25) and using lipoplexes with a charge ratio (+/−) = 10. Fig. 6 shows the results concerning antibody response of mice immunized subcutaneously with the referred formulations. The administration of lacZp25 associated with DOTAP led to an increase of the antibody titer of 2-fold of initial value after two plasmid administrations. In control mice, only a slight increase of antibody titer was detected after the first administration though not exceeding 1.4-fold of the initial titer. Statistical analyses have shown that the antibody titers for lacZp25/Lip group are significantly higher ($p < 0.001$) than those for lacZp25 and the Lip mice groups, whereas these two groups are not statistically different between them.

4. Discussion

Our study aimed at establishing the best formulation for a plasmid DNA vaccine against Maedi Visna virus, using cationic liposomes as delivery agent. A physical chemistry characterization of lipoplexes was accomplished giving insights on the most stable formulations, feasible to be further used on *in vitro* and *in vivo* experiments.

In transfection procedures *in vitro*, PBS is a buffer frequently used because of its physiologic saline concentration. Some procedures use liposomes prepared in this buffer to avoid osmotic shock when previously prepared in water or in a low ionic strength buffer. When lipoplexes are prepared with cationic liposomes hydrated in PBS (150 mM NaCl) and DNA ressuspended in the same solution, neutralization occurs at higher charge ratios, and is accompanied with a more severe aggregation. A wider range of lipoplexes charge ratios ($2 < +/- < 8$) with higher mean diameters (Fig. 3) was obtained with this saline condition. Lipoplex size variation with charge ratio generally follows a Gaussian curve similar to the obtained by us (Fig. 3). Complexes with charge ratios close to neutrality are colloidally more unstable and show an increased size, and lipoplexes with lower and higher charge ratios have approximately the same mean diameter. This dependence of lipoplex size on charge ratio was observed by other researchers in different systems (Xu et al., 1999; Kreiss et al., 1999). Accordingly, the mean diameter of lipoplexes with charge ratio 5 ranges from 1.2 to 1.6 μm and lipoplexes with charge ratio 14 is probably similar to the obtained for charge ratio 10 (~0.2 μm). Using zeta potential measurements of lipoplexes, Eastman and colleagues also verified that an increased amount of cationic lipid is required to neutralize the negative charges on a DNA cloud at high ionic strength (Eastman et al., 1997). In fact, in a first approach, by agarose gel electrophoresis, it was verified that the absence of free DNA only occurs with charge ratios (+/−) above 14 (Fig. 2), whereas in a low saline buffer at a charge ratio (+/−) = 8 no free DNA is detected (Madeira et al., 2007). However, when lipoplexes prepared in low ionic solutions are placed in a physiologic saline solution, release of DNA probably occurs to a certain extent, because under these conditions free DNA is observed at a charge ratio equal to 12 (data not shown).

Lipoplexes prepared in PBS and at charge ratios above 5, complexation efficiencies are high (96–100%) and almost invariant with the increase of charge ratio. This is probably due to aggregation of lipoplexes, also denoted by size measurements, where only small portions of DNA are not covered by liposomes. This invariance is probably due to the charge ratio range where charge neutralization of the DNA and cationic lipid occurs. Accordingly, by agarose gel electrophoresis it was possible to verify the presence of DNA at these charge ratios which corroborates the instability of these lipoplexes. Major differences are observed at lower charge ratios with smaller lipoplexes and only 23% of DNA molecules covered by the DNA (table in Fig. 3). Therefore, we may identify three different types of lipoplexes with distinct physical chemical properties that we will be further compared in *in vitro* and *in vivo* experiments: Lipoplexes with (+/−) = 1—stable and with unbounded DNA; (+/−) = 5—unstable and (+/−) = 10—more stable with a major part of DNA covered by the lipids. Different formulations may exhibit different *in vitro* and *in vivo* behaviours.

Lipoplexes with higher sizes are more unstable and are not suitable for *in vitro* or *in vivo* use (Pedroso de Lima et al., 2003), although most of the DNA is covered by the liposomes. *In vitro* transfection with smaller lipoplexes at (+/−) = 1 and 10 with 23% and 97% DNA bound to liposomes, respectively, gave rise to higher transfection efficiencies when compared to lipoplexes with (+/−) = 5. Not surprisingly, lipoplexes with higher amount of bounded DNA (charge ratio (+/−) = 10) have showed better transfection results, accordingly with cationic lipid ability to deliver pDNA into the cells and probably into the nucleus (Gao and Huang, 1995; Zabner et al., 1995).

A clear difference between *in vitro* and *in vivo* transfection efficiency was observed. In fact, a drawback of the use of cationic liposomes as non-viral vector for gene delivery is the usual lack of correlation between *in vitro* and *in vivo* results. The *in vivo* extracellular barriers are known as the major responsible for the discrepant results in both situations (Faneca et al., 2002; Templeton, 2003).

In our study, the optimal charge ratio for *in vitro* transfection was (+/−) = 10 while for *in vivo* experiments better results were obtained with a lower charge ratio (+/−) = 1, where only 23% of the DNA (table in Fig. 3) was bonded to liposomes according to our characterization studies. Other possible explanation is that the CHO cells used in *in vitro* experiments and mouse cells transfected *in vivo* may have different characteristics, which turn them more or less susceptible to be transfected, depending on the experimental conditions used.

Interestingly, each dose of DNA vaccine associated with cationic liposomes at a (+/−) = 1, administrated *in vivo*, displayed higher antibody titer than those obtained with lipoplexes at (+/−) = 10 even using a higher amount of DNA/dose in the last condition (20 μg and 50 μg, respectively). The lipoplexes were administered subcutaneously, and probably several of them entered in the blood stream. According to several authors, the blood proteins coat to lipoplexes, which may be responsible for the loss of transfection activity (Tranchant et al., 2004). On the other hand, *in vivo*, liposomes can act as adjuvants that are substances included in the vaccine formulation known to accelerate, extend or increase a specific immune response against the antigen (Vogel, 2000). When lipoplexes containing liposomes with a charge ratio (+/−) of 10 are administered *in vivo*, a cytotoxic effect may occur in the macrophages. In fact, macrophages have the ability to phagocytosis a large amount of liposomes, and are one of the major sites of liposome localization after parenteral administration (Velinova et al., 1996). Several liposomal formulations are known to be toxic to several cell lines including macrophages, and the DNA/lipid charge ratio is one of the considered parameters (Filion and Phillips, 1997). Moreover, dose, time, period of antigen availability and localization and distribution of antigen are important for immune response (Zinkernagel, 2000).

The plasmid lacZp25 is a good candidate vaccine against Maedi Visna virus, since it triggered a strong antibody response in mice. Moreover, the immunogenicity of this vaccine may be improved by the administration of lacZp25 in a solution containing liposomes at a (+/−) = 1 which may have two functions: DNA protection against nucleases and DNA transportation into the cells (Crook et al., 1996; Faneca et al., 2002) On the other hand, as an adjuvant it was previously verified that cationic liposomes also improves the specific immune response and there are several works relating the use of lipoplexes at low charge ratios in DNA vaccination (D'Souza et al., 2002; Dass, 2002).

5. Conclusions

In this work, we have developed a DNA vaccine against ovine Maedi Visna virus optimizing their immunogenity using liposomes as gene delivery vehicle. In order to predict the more effective lipoplex formulation, physical-chemistry and biological characterization of lipoplexes prepared at several charge ratios were accomplished. These studies showed that at a charge ratio of 1 (with only 23% of DNA complexed with liposomes) lower *in vitro* transfection efficiency is achieved when compared to the results obtained with lipoplexes at charge ratio 10 (with 97% of DNA covered by lipids). However, the administration of lipoplexes *in vivo* elicited a higher antibody response with (+/−) = 1. These observations were probably due to the *in vivo* extracellular barriers, such as blood proteins and to citotoxicity caused by the high amount of liposomes. Alternatively, higher expression levels due to higher transfection for a charge ratio of 10 may decrease the antibody response favouring the cellular response.

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