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Title: Delivery of recombinant vaccines against bovine herpesvirus type 1 and Babesia bovis to mice using liposomes derived from egg yolk lipids

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Abstract: Liposomes were prepared out of total egg yolk lipid extracts, and applied to deliver experimental DNA vaccines in mice. As proof of principle, the tested vaccines consisted in pCI-neo plasmids encoding BoHV-1 gD and Babesia bovis msa-2c genes. Higher numbers of mice developed an immunoglobulin G response when vaccinated with liposome-entrapped DNA as compared to naked DNA, with significant differences only for the msa-2c groups. The antibody titres reached were similar in all cases. These results indicate that, likely depending on the targeted antigen, liposomes made out of non purified egg yolk lipid extracts are able to improve the immunological response to DNA vaccines, and can represent a significant contribution to vaccinology in the veterinary field.

Short Communication

Delivery of recombinant vaccines against bovine herpesvirus type 1 and *Babesia bovis* to mice using liposomes derived from egg yolk lipids

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Abstract

Liposomes were prepared out of total egg yolk lipid extracts, and applied to deliver experimental DNA vaccines in mice. As proof of principle, the tested vaccines consisted in pCI-neo plasmids encoding BoHV-1 gD and *Babesia bovis* msa-2c genes. Higher numbers of mice developed an immunoglobulin G response when vaccinated with liposome-entrapped DNA as compared to naked DNA, with significant differences only for the msa-2c groups. The antibody titres reached were similar in all cases. These results indicate that, likely depending on the targeted antigen, liposomes made out of non purified egg yolk lipid extracts are able to improve the immunological response to DNA vaccines, and can represent a significant contribution to vaccinology in the veterinary field.

Keywords: DNA vaccine; Egg yolk liposome

Injection of plasmid DNA can elicit humoral and cell-mediated immune responses against a wide variety of encoded antigens. DNA uptake by muscular or other cells is probably followed by expression and extracellular release of the antigen, which can then be taken up by antigen presenting cells (APCs). Direct uptake of DNA by APCs is also possible. Due to the localisation and post-translational modifications of the encoded antigens, DNA vaccines are likely to elicit immune responses that better mimic a natural infection than bacteria-expressed antigens. Experimental DNA vaccines have been produced against several pathogens, and there are currently two licensed DNA vaccines of veterinary or productive importance (Powell, 2004; Lorenzen and LaPatra, 2005).

Since DNA internalisation is hampered by poor uptake efficiency and degradation by deoxyribonucleases present in the interstitial fluids, encapsulation in liposomes is frequently used to increase the efficiency of DNA vaccines. However, the high cost of required purified phospholipids is particularly constraining for the preparation of vaccines of veterinary use.

We have explored the usefulness of liposomes prepared out of an inexpensive source of lipids, egg yolk, for the entrapment and delivery of DNA. As a proof of principle, we tested the modulation effects of these liposomes on the humoral response elicited by DNA encoding two different antigens of pathogens of veterinary importance: glycoprotein D (gD) of bovine herpesvirus type 1 (BoHV-1) and the merozoite surface antigen-2c (MSA-2c) of *Babesia bovis*.

BoHV-1 infects the respiratory and genital tracts of domestic cattle, with harsh economic impact worldwide. Vaccination of bovines with plasmids encoding gD, the main

BoHV-1 vaccine candidate, induced varying degrees of immune protection upon challenge, depending on the adjuvant of choice (van Drunen et al., 1998; Petrini et al., 2011).

B. bovis is a tick-transmitted protozoon that severely affects cattle in the tropics. There is general consent that there is a need for the development of subunit formulations that replace the currently available live vaccines against this haemoparasite (Suarez and Noh, 2011). Vaccination of cattle with recombinant (r) MSA-2c, a conserved surface antigen with neutralisation-sensitive B-cell epitopes, elicited a long-lasting humoral response (Wilkowsky et al., 2003), but only mild protection against challenge (Alvarez et al., 2010). Alternative formulations such as DNA delivery for this and other antigens are worth exploring in future vaccine formulations for *B. bovis*.

For liposome preparation, egg yolks from eggs weighing at least 58 g were separated from whites and homogenised by stirring. Total lipids were extracted according to Bligh and Dyer (1959), weighed and analyzed by thin layer chromatography, followed by densitometry measurements. The percentage composition consisted of 21% phosphatidylcholine (PC), 25% phosphatidylethanolamine, 10% cholesterol and 10% triacyglycerol, as main components. A chloroform solution of egg yolk lipid extract was then prepared, gassed with nitrogen and stored at -20 °C.

The complete open reading frames of gD (GenBank AJ004801, 1121 base pairs, bp) and msa-2c (GenBank AY052542, 798 bp) were amplified by PCR (Zamorano et al., 2002; Wilkowsky et al., 2003). Amplicons were cloned into pCI-neo (Promega), amplified in *E. coli* BL21 competent cells and purified using a Maxiprep kit (Wizard Plus, Promega). Non recombinant (nr) pCI-neo plasmid was also amplified and purified the same way.

Plasmid preparations were complexed to egg yolk lipid liposomes (0.22 mg lipid, containing approximately 0.032 μmoles PC, per μg plasmid), following the dehydration-rehydration method of Gregoriadis et al. (2000). After ultracentrifugation of liposome-entrapped DNA, absorbance at 260 nm (A_{260}) by spectrophotometry showed that the amount of non encapsulated plasmid remaining in the supernatants was less than 10%. The precipitated liposomes were suspended in phosphate buffered saline (PBS) and used immediately. A heterogeneous suspension of 0.2 to 0.8 μm diameter vesicles was obtained, as observed by electron microscopic examination (Appendix A: Supplementary Fig. 1).

Four groups of 10 Balb-c 2-month-old male mice were subcutaneously inoculated at days 0 and 15 with 25 μg gD-pCI-neo or msa-2c-pCI-neo, either naked or encapsulated in egg yolk liposomes, in a final volume of 0.2 mL. Additionally, groups of five mice were inoculated with either naked or liposome-complexed nr pCI-neo plasmid (25 μg), as negative controls. Mice were bled at 0, 15 and 30 days post inoculation (dpi) and sera were separated and stored at -20 °C until use. Total immunoglobulin G (IgG) titres against rgD or rMSA-2c were measured by indirect ELISA, as previously described, using murine sera against these antigens as plate controls (Zamorano et al., 2002; Wilkowsky et al., 2003). Titres were calculated as the $\log_{10}$ of the maximum dilution that gave an A_{492} value higher than the average of the corresponding negative values + 3 standard deviations (SD). Mice were considered to be immunologically responsive when IgG titres were ≥ 2. The protocol for experiments with mice was retrospectively approved by the Institutional Committee for the Care and Use of Experimental Animals (CICUAE, CICVyA-INTA, 21/2012, 15/08/2012).

At 15 dpi, 16 vs. 2 mice had developed an IgG response in the liposome-encapsulated vs. naked DNA groups; while at 30 dpi, responsive mice were 16 vs. 11, respectively (Fig. 1). Chi square (χ^2) contingency test with 1 degree of freedom (df = 1) showed that differences between mice numbers were highly significant in the msa-2c-vaccinated groups, both at 15 (P <0.0003) and 30 dpi (P <0.007). Differences among the gD groups, on the other hand, were non significant according to this test. IgG titres obtained with both types of formulations were similar (Fig. 1).

Thus, encapsulation in egg yolk liposomes can increase the reliability of the immunoglobulin response elicited by DNA vaccination in mice. Interestingly, this effect seems to depend on the tested antigen. Future experiments will be devoted to assay this methodology in the bovine, the natural host of these pathogens.

The method used involves low cost reagents, regular laboratory equipment and an easily up-scalable procedure. These characteristics make this system particularly suitable for veterinary applications, although its use in human medicine should also be explored. Delivery of drugs and other substances using these liposomes is currently under investigation.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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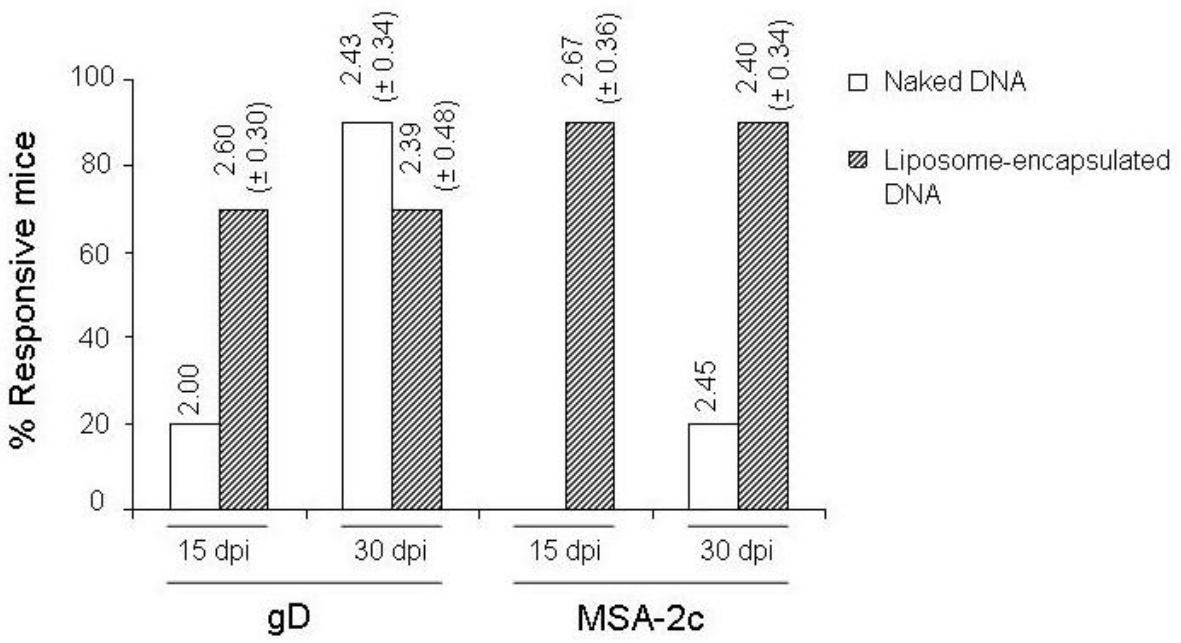
Figure legends

Fig. 1. Humoral responses of mice vaccinated with plasmids encoding gD or MSA-2c, either naked or in liposomes. Results are shown as percentages of responsive mice, i.e. those that developed serum IgG titres ≥2 (which corresponds to a serum dilution of 1/100), in each group of 10 mice at 15 and 30 dpi, after receiving one or two doses, respectively, of each DNA vaccine preparation. The average IgG titres (± standard deviation when appropriate) for each group are shown above each bar. Titre variations were <20% in all cases. Comparisons of mean titres between groups that had three or more responsive mice by Student's *t* test showed no significant differences (naked vs. liposome-gD at 30 dpi; 15 vs. 30 dpi obtained with liposome-gD or liposome-msa-2c).

Figure


% Responsive mice
100
80
60
40
20
0
2.00
2.60 (± 0.30)
2.43 (± 0.34)
2.39 (± 0.48)
2.67 (± 0.36)
2.45
2.40 (± 0.34)
15 dpi
30 dpi
15 dpi
30 dpi
gD
MSA-2c
Naked DNA
Liposome-encapsulated DNA

Optional e-only supplementary files

Click here to download Optional e-only supplementary files: YTVJL-D-12-002775-Appendix-A-SupplementaryFile-Edited-AWP-10

Revision Note
Ms. YTVJL-D-12-002775

The following changes have been introduced to the manuscript, according to the comments of Dr. Adrian Philbey:

1) Please upload Fig. 1 as a separate figure file according to the journal requirements, removing the heading "Fig. 1" from the top.
Answer: This has been done

2) The IgG titres (+/- standard deviation) above each bar on the chart should be reformatted so that the font type is consistent with text used elsewhere in the graph. Please also use an appropriate Word symbol for "+/-".
Answer: The font in all texts of Figure 1 has now been changed to Arial, in either size 12 or 16. The symbol +/- has been changed to ±. In addition, the legends corresponding to the IgG titers are now in vertical orientation, to avoid the "pixelling" that occurred in the diagonal orientation.

3) Please remove the red border from around Supplementary Fig. 1.
Answer: This has been done

4) Check this version carefully for accuracy and completeness before your paper is ready to be accepted for publication.
Answer: We have revised the ms, and the only change that we have introduced is the replacement of the > symbol for ≥ in lines 105 and 185.

Secretaría de Agricultura, Ganadería, Pesca y Alimentación
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2012 - Año de Homenaje al
Dr. D. Manuel Belgrano

Hurlingham, October 15, 2012

Dr. Andrew Higgins
Editor-in-Chief
The Veterinary Journal

Dear Dr. Higgins,

On behalf of my co-authors I hereby submit a revised version of Ms. YTVJL-D-12-002775: "Delivery of recombinant vaccines against bovine herpesvirus type 1 and *Babesia bovis* to mice using liposomes derived from egg yolk lipids". In this version, we have addressed the last comments of our editor, Dr. Adrian Philbey, as described in the "Revision Note". No additional changes were required by the reviewers.

We want to take the opportunity to gratefully acknowledge the detailed revision carried out by the reviewers and the outstanding editing work performed by Dr. Philbey, which has considerably improved the presentation of our data.

Yours sincerely,

Monica Florin-Christensen, Ph.D.
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