Integrity and Activity Evaluation of *S. equi* Antigens Encapsulated in Liposomes

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INTRODUCTION AND OBJECTIVES

Strangles is a deadly respiratory tract disease that affects the nasopharynx and draining lymph nodes of *Equidae*. *Streptococcus equi* subsp. *equi* (*S. equi*) is the causative agent of strangles, being easily transmitted among animals.[1] This is an extended disease, easily leading to large outbreaks and having high consequences at the economical level.[2]
S. equi M like protein (SeM) is believed to be the basis of virulence, however, horse vaccination with SeM associated to different adjuvants, did not protect animals against infection with *S. equi*.[2] On the other hand non-aggregated *S. equi* enzymatic extract protein-adsorbed PCL microspheres enhanced serum specific IgG, IgG1 and IgG2a antibody responses. [2,3]
Intranasal vaccination seems to be a good choice to reach protective immunity, once main access for *S. equi* is the nasal mucosa, in association with the vantage of being easily administrated in *Equidae*.[2]
On the other hand, liposomes have shown to be effective in inducing mucosal immune responses following intranasal administration, protecting the antigens against enzymatic degradation and enhancing the uptake by specialized cells.[3]
In this work, liposome formulation encapsulating *S. equi* antigens was optimized and characterized. Protein integrity after encapsulation was assessed. Lipossomal adjuvant capacities were tested *in vivo* in order to evaluate immune response to the particulate system.

MATERIALS AND METHODS

Materials Egg-phosphatidylcholine (PC) was obtained from Lipoid, Ludwigshafen, FRG. Cholesterol and Stearylamine were purchased from Sigma. Enzimatic extract obtained from *Streptococcus equi* subsp. *equi* (strain LEX) as described in Florindo et al- (2008).
Animals Female BALB/c mice (n=5/group), 6–8weeks old provided with food and drink *ad libitum*, were used in the *in vivo* studies, which were performed in strict accordance with Directive of 24 November (nº 86/609 EEC), the Portuguese laws D.R. nº 31/92, D.R. 153 I-A 67/92, and all following legislations.
Liposome preparation Positively charged liposomes were prepared according to a method previously described by Corvo et al (2002) with slight alterations. Briefly, thin films were obtained by rotary evaporation of a mixture of the appropriate amounts of PC:Chol:SA in a molar ratio of 7:3:1 in chloroform, with a lipid concentration of 40 mM. The lipid films were kept under a nitrogen stream until being completely dried. The film was than dispersed in an enzymatic extract solution, or BSA solution in water. After lyophilization overnight the lyophilized powder was hydrated with 0.145 M NaCl/10 mM citrate buffer pH 5.6. Liposomes obtained were extruded sequentially through polycarbonate filters with pore sizes of 0.8, 0.4, 0.2 µm. Nonencapsulated protein was separated from the liposome by dilution and ultracentrifugation at 300,000xg for 180 min at 10ºC in a Beckman L8-60M ultracentrifuge. Finally, liposomes were dispersed in a 0.145 M NaCl/10 mM citrate buffer pH 5.6.
Liposome characterization Mean particle size was measured by dynamic light scattering with a Zetasizer, model Nano S (Malvern) and Zeta potential measurement using laser Doppler electrophoresis with a Zetasizer model 2000 (Malvern).
Phospholipid concentration was determined as described by Rouser et al (1970) or with Sprinreact Phospholipid Kit when formulation was dispersed with enzymatic extract in PBS buffer.
Protein was determined with a modified Lowry method with prior disruption of liposomes with Triton X-100 and sodium dodecylsulphate according to Wang, et al (1975).
Structural integrity of S. equi antigens The molecular weight integrity of encapsulated *S. equi* antigens in liposomes was determined by polyacrylamide gel electrophoresis under denaturating condition (SDS-PAGE) by comparision with molecular weight reference marker (molecular weight 6.0-181kDa, Invitrogen, UK). Samples were loaded onto NuPAGE 10% Bis-Tris Gel (Invitrogen, UK). Proteins were visualized by Coomassie blue staining (SimplyBlue™ SafeStain solution, Invitrogen, USA).
Immunisation studies Two groups of female BALB/c were immunized by intransal route on day 1 and boosted on day 22, by instillation using a micropipette tip (25 ml per nostril). Controls consisted of empty liposomes.Blood samples were collected every 15 days from the tail vein.
Quantification of serum specific antibodies IgG, IgG1 and IgG2a Serum *S. equi* specific IgG, IgG1, IgG2a antibodies were detected by ELISA as previously described by Florindo et al (2008) with slight alterations. In brief, 96 well ELISA microplates (Microlon®, Greiner bio-one, Germany) were coated overnight at 4ºC with 10µg/ml SeM protein in carbonate buffer (pH=9,6). After washing with 0,05% (v/v) solution of Tween 20 (Sigma—Aldrich Co. UK) in PBS, pH=7,4 (TPBS) microplates were incubated at 37ºC with a 5% (m/v) skim milk solution in TBS (Merck, Germany) for 1h. The plates were then washed with TPBS and pool serum samples at a 20-fold dilution were added to wells. Twofold dilutions were made along the plate and then incubated

for 90 min at 37ºC. Plates were again washed with TPBS and antibody binding was detected adding to each well goat anti-mouse antibody-horseradish peroxide conjugate diluted in the skim milk solution (IgG (Serotec, UK) diluted 1:1000, IgG1 (Serotec, UK) diluted 1:2000, IgG2a (Serotec, UK) diluted 1:2000). The plates were incubated for 90 min. at 37ºC and washed with TPBS. Quantification of specific antibodies was made with SigmaFAST™ OPD Kit (Sigma,USA), measuring absorbance at 490nm after stoping reaction with H_2SO_4.

RESULTS AND DISCUSSION

Among different combinations of lipidic compositions, PC:Chol:SA with a molar ratio of 7:3:1 showed to be the most stable and with higher encapsulation efficiency (E.E.) described by equation 1.

$$E.E(\%) = \frac{[protein]_{final}}{[lipid]_{final}} / \frac{[protein]_{inicial}}{[lipid]_{inicial}} \quad \text{(eq.1)}$$

Preliminary E.E. studies carried out with BSA revealed an E.E. of around 60% when the initial BSA concentration was 5mg/ml. The mean diameter of the final liposomal formulations were 0.20 µm with a polydispersion index (PDI) of 0,10.

When *S. equi* antigens were used, E.E decreased to 34% for an initial protein concentration of 6mg/ml. No changes were observed for the mean diameter of the particles remaining in order of 0.20µm. Liposomes encapsulating enzymatic extract remained positively charged, with a zeta potencial of +18 mV.

The maintenance of protein integrity after encapsulation was assessed through SDS–PAGE (Fig. 1). Once SeM is a 58 kDa M-like protein it may be identified near the 56 kDa marker.

The migration pattern of native *S. equi* antigens is identical to those encapsulated in liposomes before and after extrusion, suggesting that protein molecular weights were not affected by liposomal encapsulation.

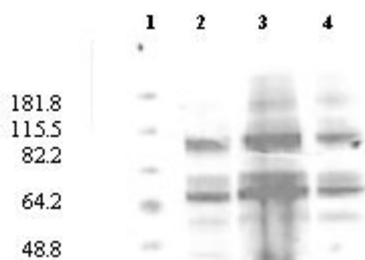

Figure 1 - SDS–PAGE (10% gel) of *S. equi* enzymatic extract solutions before and after encapsulation. Lanes: (1) standard molecular weight markers; (2) Enzimatic extract SeM (3) Liposomes with antigens prior to extrusion (4) Liposomes encapsulating antigens after extrusion

After vaccination, immugenicity of the liposomes entrapping *S. equi* antigens is confirmed by the growing systemic levels of specific IgG, IgG1 and IgG2a (Fig. 2). No *S equi*-specific IgG antibodies were detected in serum of animals vaccinated with empty liposomes (data not shown).

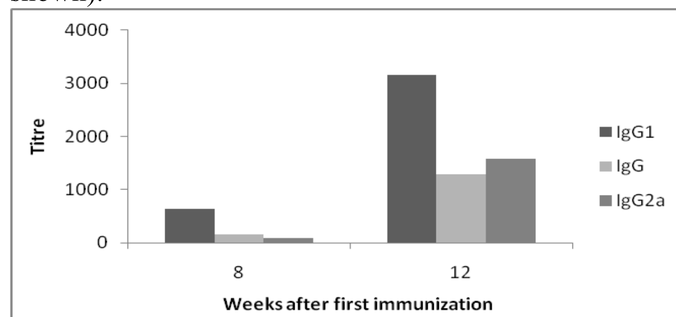

Figure 2 – Serum anti-*S equi* specific IgG, IgG1 and IgG2a titres induced after mice immunizarion by i.n. route with liposomes encapsulating *S. equi* antigens.

The ratio between specific IgG titres (IgG2a/IgG1) becomes more equilibrated along the time (0,13 in the 8th week and 0,50 in the 12th week) indicating a balance between humoral and cellular immune response.

CONCLUSION

In conclusion, these results confirm, the immunological adjuvant properties of liposomes, which seem a particularly t suitable alternative formulation for *S. equi* antigen delivery by the intranasal route, thus facilitating a well equilibrated immune response.

REFERENCES

[1] Florindo HF et al. (2009) *The enhancement of the immune response against S. equi antigens through the intranasal administration of poly- 3 -caprolactone-based nanoparticles* Biomaterials (30) 879-891

[2] Florindo HF et al. (2009) *New approach on the development of a mucosal vaccine against strangles: Systemic and mucosal immune responses in a mouse model.* Vaccine (27) 1230-1241

[3] Florindo HF et al. (2008) *Streptococcus equi antigens adsorbed onto surface modified poly-ε-caprolactone microspheres induce humoral and cellular specific immune responses.* Vaccine (26) 4168-4177

[4] Baca-Estrada ME et al (2000) *Intranasal immunization with liposome-formulated Yersinia pestis vaccine enhances mucosal immune responses* Vaccine (18) 2203-2211

[5] Corvo,M.L et al (2002) *Superoxide dismutase entrapped in long-circulating liposomes: formulation design and therapeutic activity in rat adjuvant arthritis.* Biochimica Biophysica Acta-Biomembranes (1564) 227-236.

[6] G. Rouser et al (1970) *Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipidsby phosphorus analysis of spots.* Lipids (5) 494–496

[7] C.-H. Wang, et al.(1975) *Lowry determination of the protein in the presence of Triton X-100* Anal. Biochem. (63) 414– 417