In vitro study of protein release from AFCo1 and implications in mucosal immunisation

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Adjuvant Finlay Cochleate 1 (AFCo1) is a Proteoliposome-derived cochleate obtained from Neisseria meningitidis serogroup B. Transformation of proteoliposomes into AFCo1 potentiates the immune response on Neisseria antigens when it is administered by intranasal or intragastric (i.g) routes. However, the i.n route has been demonstrated to be more effective. The aim of this work is to evaluate in vitro the protein release from AFCo1, in simulated gastric fluid (SGF) or simulated nasal fluid (SNF) using a microdissolution test and to provide support for the results found when AFCo1 was administered by i.g or i.n routes in BALB/c mice. Results showed that dilution of AFCo1 in simulated gastric fluid affects the delivery of Neisseria protein antigens because they were released from cochleate structures faster than when simulated nasal fluid was used. In conclusion, conditions simulating gastric environment affect the delivery of protein antigens from AFCo1 and this result could partially explain why i.n administration is more effective in vivo than i.g immunisation.

Keywords: Cochleate, adjuvant, proteoliposome, *Neisseria*.

Introduction

Most pathogens either invade the body or establish infection in mucosal tissues and represent an enormous challenge for vaccine development by the absence of good mucosal adjuvants. AFCo1 (Adjuvant Finlay Cochleate 1) is an adjuvant microparticle derived from *Neisseria meningitidis* serogroup B proteoliposome (PLn) (1). It contains several immunopotentiator and antigenic molecules such as lipopolisacharides (LPS) and outer membrane porins (2).

Several studies have demonstrated the AFCo1 adjuvant effect on *Neisseria*, *Leishmania* and ovalbumin antigens administered either by parenteral or mucosal route (2,3). Recently, del Campo *et al*. (2009) reported that AFCo1 elicited immune responses against *Neisseria* antigens by intranasal (i.n) or intragastric (i.g) route of immunisation (3) and they also found that i.n was more immunogenic. It was suggested that stomach conditions of low pH and proteases affect delivery or antigen integrity (3). Therefore, the aim of this work was to evaluate the *in vitro* release of protein antigens from AFCo1 using a microdissolution method with fluids simulating i.g or i.n conditions.

The suitability of predicting *in vivo* behavior of drug products using simulated fluid has been demonstrated in several *in vitro-in vivo* correlation studies (4, 5). However, this correlation may depend on multiple factors such as dosage form, administration route and the test used to evaluate the release of the encapsulated material (6). The dissolution test is the most used and recognised by pharmacopeia to determine the solubility and *in vitro* release of active molecules in formulation (6). But this method demands large amounts of drug, and biological active principles are usually only available in low quantities. Therefore, we selected a miniaturized flask method described by Glomme et al. (2005) (7), adapted it to our laboratory conditions, referring to it as a microdissolution test.

Materials and Methods

Preparation of AFCo1 structures

PLn was provided by the production vice-presidency of Finlay Institute. PLn was resuspended and adjusted to 1 mg/mL in a buffer containing 30 mmol/L Tris, 3 mmol/L ethylenediaminetetra-acetic acid and 1.5% (w/v) sodium deoxycholate. AFCo1 formation was performed as previously described by Pérez O et al (2008) (1). Briefly, detergent was eliminated and calcium (Ca^{2+}) was incorporated at the same time using a rotary dialysis method for lab scale. The Ca^{2+} formation buffer was prepared with 30 mmol/L Tris, 100 mmol/L NaCl and 5 mmol/L $CaCl_2$ at pH 7.4.

The process was marked by the appearance of a white suspension as cochleates were formed. The efficiency of the incorporation process was estimated by comparing the protein quantities of the precipitate and the supernatant by Lowry.

AFCo1 structure was observed by light microscopy using an Opton Standard 25 microscope and the average length size of 200 particles was determined using a gradation scale on an ocular lens. Additionally, AFCo1 was observed by transmission electron microscopy as described in the next section.

Negative Staining and Transmission Electron Microscopy (TEM). These experiments were carried out as described by Xiaozhong *et al* (2004)(8). Briefly, Formvar/Carbon-coated 200 mesh copper grids were glow discharged. Cochleate samples were resuspended in 30 mM Tris buffer and dried using filter paper to a thin layer onto the hydrophilic support film. Twenty microlitres of 1% (v/v) aqueous methylamine vanadate stain (Nanovan; Nanoprobes, Stony Brook, NY, USA) was applied and excess moisture removed with filter paper. Dried samples were imaged with a LEO 912 energy filtering transmission electron microscope operating at 120 kV. Contrast enhanced, zero-loss energy filtered digital images were recorded with a 14 bit /2 K Proscan CCD camera.

Dissolution test

Media Preparation. Simulated fluids (SF) were prepared according to USP 29/NF 24 (9). Briefly, Simulated Gastric Fluid (SGF): 34.2 mM of sodium chloride and 0.09mM of pepsin were dissolved in water with hydrochloric acid 0.2 M to pH 1.2. No references on Simulated Nasal Fluid (SNF) were found in specialized books (USP or Martindale) so we used a formula for Simulated Intestinal Fluid without pancreatin, which is similar to the physiological conditions of nasal mucosa (10). Then, 50.3 mM of potassium dihydrogen phosphate and 22.4 mM of sodium hydroxide were dissolved in water to final pH of 6.8.

Microdissolution method for release studies. A variation of the miniaturized shake-flask method (7) was employed to evaluate protein release from AFCo1 when treated with SGF or SNF. Five micro-centrifuge tubes were prepared for each *in vitro* simulated condition and 2 mL of dissolution sample was added to the simulated fluids and AFCo1 (5:1) v/v.

The samples were shaken (100 rpm) at 37 °C in an orbital shaker (Heidolph Polymax 1040). At predetermined time intervals (0, 15, 30, 60 and 120 minutes), samples were centrifuged at 10 000 rpm for 5 min. The supernatant was collected and kept at 4 °C until evaluation. The pellet was re-suspended up to the same volume with calcium formation buffer and the protein concentration was evaluated by Lowry assay (1).

Immunization and sample conection

Groups of five female BALB/c mice were inoculated by i.n or i.g route with AFCo1 (100 μg per dose per mouse). Mice received three inoculations for mucosal routes: 20 μL of AFCo1 (10 μL per nostril) by the i.n route without anesthesia

or 100 μL of AFCo1 by i.g gavage. Placebo groups were immunized with 20 μL or 100 μL of phosphate buffered saline (PBS) by i.n or i.g route respectively. Saliva samples were taken by stimulating salivation using an intraperitoneal injection of 50 μL of 0.5% pilocarpine. This samples were inactivated for 15 min at 56 °C and centrifuged at 10 000 g for 10 min. For serum collection, animals were bled by retro-orbital puncture 14 days after the last immunisation, and the samples were centrifuged at 5000 g for 10 min. and stored at -20 °C for subsequent analysis. Animals were housed at the Finlay Institute animal facility and all experiments were performed with approval of the Finlay Institute Ethics Committee.

Enzyme-linked immunosorbent assay (ELISA) for PLn specific IgA and IgG elicited by AFCo1 administration

Specific IgG anti-PLn antibodies in serum samples and IgA anti-PLn antibodies in saliva were measured by indirect ELISA using polystyrene 96-well plates MaxiSorp coated with PLn (100 μL *per* well) at 20 μg/mL in PBS buffer (0.15 mol/L, pH 7.3) at 4 °C overnight, and blocked with 1% (w/v) BSA in PBS (blocking solution) for 1 h at room temperature.

Serum samples were diluted 1:100 and saliva 1:2 in blocking solution and incubated for 1 h at 37 °C. Anti-mouse IgG or IgA peroxidase-conjugated antibodies (Sigma) were added (100 ìL *per* well) at 1:2500 dilution in blocking solution and incubated for 1 h at 37 °C. Bound antibodies were detected with 100 μL *per* well of the substrate-chromogen mixture (o-phenylenediamine and hydrogen peroxide in citrate-phosphate buffer, pH 5). The reaction was stopped by adding 50 μL of sulphuric acid at 2 mol/L and optical density at 492 nm was measured in a microplate reader (Titertek, Multiskan Plus; Labsystem).

All incubation steps were followed by three washes with PBS containing 0.05% Tween-20 (v/v). ELISA results were expressed as units per mL, referred to an in-house standard. The means and standard errors of the mean (SEM) of at least three different experiments were analysed.

Statistical Methods. Data from *in vitro* experiment was analyzed by a two-way ANOVA followed by Bonferroni post-hoc test. Statistical analysis of *in vivo* data was carried out with a Student's *t* test. The samples were first subjected to Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lillie for p-value. All the statistical analyses were performed using Graph Pad Prism 4 software (CA, USA).

Results

AFCo1 formation and characterization

PLn was transformed into AFCo1 using the dialysis rotary method. In this process, calcium interacts with phospholipids, proteins and LPS in the PLn (Fig. 1A) forming a microtubular

structure as observed in Figure 1B. The length of the AFCo1 measured from TEM micrographs was 13.5 μm and the width was 2.6 mm. Similarly, the average length of the cochleates was measured by light microscopy (15.6 μm ± 4.2 μm).

In vitro study of protein release from AFCo1

A microdissolution test was used to evaluate protein release from AFCo1 in two different simulated fluids. More than 40% of proteins in AFCo1 were released from the samples incubated in SGF or SNF in the first 15 min of the test, but were faster released from AFCo1 when treated with the SGF (Fig. 2). Once the test started, significant differences were observed between treatments at 15, 30, 60 and 120 min (Fig. 2). After two hours of treatment with simulated fluids, the AFCo1 in SGF released more than 80% of proteins but more than 40% of protein was still encapsulated in the AFCo1 dissolved in SNF as shown in Figure 2.

Evaluation of immune response induced i.g *vs* i.n administration of AFCo1

BALB/c mice were immunised by i.n or i.g route with 100 mg of AFCo1 using a three dose schedule (0, 7 and 14 days). Figure 3 shows that i.n immunisation elicited higher specific IgA (Fig. 3A) and IgG (Fig. 3B) antibodies responses against antigens from *N. meningitidis* B in the PLn (p<0.01). PLn is the raw material used to obtain the AFCo1 and contains the same *Neisseria* antigens than AFCo1.

Discussion

Mucosal vaccine strategies against bacterial infections based on particulate non living delivery systems are an important strategy in the design of adjuvants and vaccine formulations (11), not only because they represent a pain free alternative, but also because they provide the potential for both mucosal and systemic protection. AFCo1 has been used as a mucosal adjuvant with several antigens (2, 3). In particular, i.n immunisation has been more immunogenic than i.g (3), but reasons ruling this behaviour are still unknown. AFCo1 is a complex tubular microparticle formed by the interaction of calcium with negatively charged phospholipids, LPS, and proteins in the PLn structure (1, 2).

They have a defined multilayered structure consisting of a solid, lipid bilayer sheet rolled up in a spiral like shape as represented in Figure 1A. AFCo1 has high amounts of PorA and PorB *Neisseria* proteins (2).

These also represent important antigens within PLn which is the core of the Cuban vaccine against *N. meningitidis* serogroup B and C, VA-MENGOC-BC®. At the same time, PorA and PorB may behave as immunostimulators to activate dendritic cells (DC) through interaction with toll like receptor (TLR)-2 (12), as well as LPS which is considered a potent activator of TLR-4 (12).
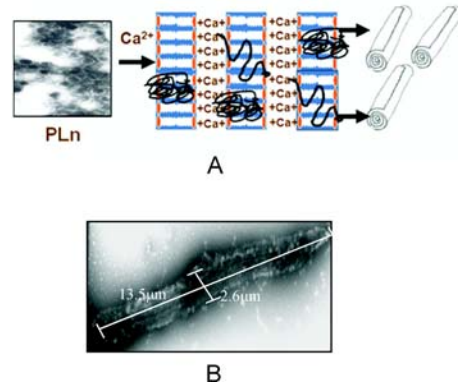

Fig. 1. AFCo1 formation. (A) Schematically represents the interaction between calcium and PLn (TEM). The TEM micrograph in (B) Shows the microtubular structure of AFCo1.
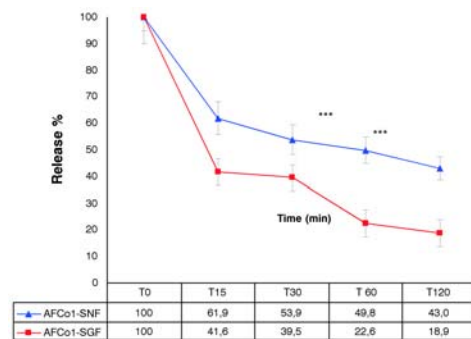

Fig. 2. Dissolution test. A microdissolution test was carried out to evaluate the release of proteins from AFCo1 over the time in two different fluids: SNF (triangles) and SGF (squares). Each time point was evaluated by duplicate and the results of three individual experiments are shown (Mean of protein released ± SEM). The statistical analysis was performed by a two-way ANOVA followed by Bonferroni post-hoc test using Graph Pad Prism 4 software. Asterisks represent (***) significant differences p<0.001.
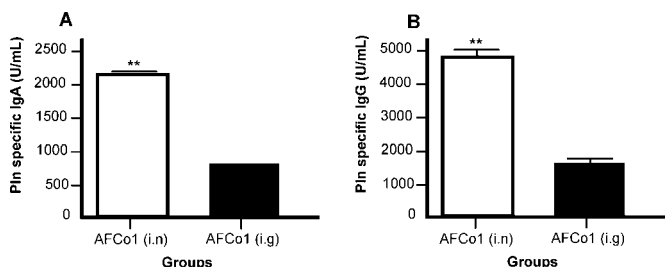

Fig. 3. Immune response induced by AFCo1 administered by mucosal route. Groups of female BALB/c mice (n = 5) were i.n or i.g immunised with AFCo1 using a three doses schedule (0, 7 and 14 days). The PLn-specific IgA levels in saliva (A) and PLn-specific IgG in sera (B), were determined using a PLn-specific ELISA. Data are expressed as Units/mL+ SEM from three independent experiments. Placebo effect was subtracted for each group and means were compared using Student's t test. Asteriks (**) represent statistic significance (p<0.01). The statistical analysis was performed using Graph Pad Prism 4 software.

An important issue for the development of an effective mucosal vaccine is whether DC within mucosal epithelia are involved in priming CD4 and CD8 effector T cells. The efficiency of oral immunisation with protein antigens to prime CD4 and CD8 T cell responses is hampered by antigen dilution/degradation in the gastrointestinal milieu (13). The nasopharyngeal mucosa offers several advantages for *in vivo* stimulation because inductive sites are mostly found in the Waldeyer's ring, a region where lymphoid tissue is less dispersed than limphoid tissue (Peyer's patch) in gastrointestinal compartments (14).

Therefore we used an *in vitro* microdissolution test to evaluate if the release of AFCo1 protein antigens/immunostimulators was affected by conditions that simulate i.g environment. Firstly, the amount of protein released over time from AFCo1 was evaluated with different fluids simulating i.n or i.g immunisation.

We then separated the unbound AFCo1 components by a brief centrifugation step instead of filtration using a Whatman UniPrep filter chamber as recommended by Glomme, et al. (2005) (7) because previous experience working with AFCo1 suspensions has demonstrated that the centrifugation step is sufficient to evaluate the incorporation of proteins into AFCo1 (3).

Results showed that proteins were released faster from samples treated with SGF, although more than 40% of proteins from samples treated with SNG or SGF were released in the first 15 min of dissolution. This result indicates that dissolution may have an important effect at the nasal and gastric compartments *in vivo* in the early moments of exposure of the cochleates to body fluids. Miclea et al (2007) found similar results when they evaluated *in vitro* release of recombinant proteins encapsulated in cochleates prepared using commercial phospholipids (15).

It seems that aqueous media favours unrolling of the AFCo1 structure due to a chemical equilibrium between the calcium forming the structure and that released into the fluid. In addition, the sample treated with SGF showed a higher percentage of protein released than that treated with SNF, probably because the acid environment may also favour the solubilisation of calcium precipitates in organic tissues (16). The i.g environment may also contribute to faster disassembly of AFCo1 and exposure of the antigenic and immunostimulator molecules to degradative effects of enzymes and low pH of the stomach.

Additionally, the results of mucosal immunisation (Fig. 3) confirm previous results obtained by del Campo, et al (3) and show significant differences between mucosal and systemic immune responses induced in mice immunised by i.n or i.g routes. However, how much the immunogenic and immunostimulator molecules in AFCo1 have been affected *in vivo* is a question that needs to be answered.

In general, this study demonstrates that a microdissolution test is an effective way to evaluate the release of proteins from AFCo1. This method may be used to determine the release of molecules incorporated in AFCo1, other than Neisseria proteins and also to evaluate cochleates derived from different bacteria proteoliposomes, such as AFCo2 (17). These results contribute to understand the action mechanism of AFCo1.

Acknowledgments

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Estudio *in vitro* de liberación de proteínas de AFCo1 y sus implicaciones en la inmunización mucosal

Resumen

El cocleato adyuvante Finlay (AFCo1) 1 es derivado de un proteopolisoma de *Neisseria meningitidis* serogrupo B. La transformación de los proteoliposomas en AFCo1 potencia la respuesta inmune de los antígenos de *Neisseria* cuando se administra por vía intranasal o intragástrica. Se ha demostrado, sin embargo, que la vía intranasal es más efectiva. Los objetivos de este trabajo fueron evaluar *in vitro* la liberación de proteínas del AFCo1 en líquido nasal o gástrico simulado, usando para ello la prueba de microdisolución y apoyar los resultados obtenidos cuando se administró AFCo1 por vía intranasal o intragástrica en ratones BALB/c. Los resultados demostraron que la dilución de AFCo1 en líquido gástrico o nasal simulado afecta la distribución de los antígenos de proteína de *Neisseria*, ya que estos se liberaron de las estructuras de cocleatos más rápido cuando se utilizó líquido nasal. Se concluyó que las condiciones que simulan el entorno gástrico afectan la distribución de los antígenos de proteínas de AFCo1 y este resultado puede explicar parcialmente porqué la administración intranasal es más efectiva *in vivo* que la inmunización intragástrica.

Palabras clave: Cocleato, adyuvante, proteoliposoma, Neisseria.