Methods 49 (2009) 309–315

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Intranasal administration of proteoliposome-derived cochleates from *Vibrio cholerae* O1 induce mucosal and systemic immune responses in mice

Reinaldo Acevedo [a,*], Adriana Callicó [a], Judith del Campo [a], Elizabeth González [a], Bárbara Cedré [a], Lissette González [c], Belkis Romeu [a], Caridad Zayas [a], Miriam Lastre [a], Sonsire Fernández [a], Reynaldo Oliva [b], Luis García [a], José Luis Pérez [a], Oliver Pérez [a]

[a] Immunology Department, Bacterial Vaccines Division of Finlay Institute, Havana, Cuba
[b] Animal Models Department, Bacterial Vaccines Division of Finlay Institute, Havana, Cuba
[c] Pharmacy and Food Institute, Havana University, Havana, Cuba

ARTICLE INFO

Article history:
Accepted 25 March 2009
Available online 21 June 2009

Keywords:
Cochleates
Adjuvant
Vaccine
Proteoliposomes
Vesicle
Intranasal
Mucosal
Vibrio cholerae
Mice

ABSTRACT

Conservative estimates place the death toll from cholera at more than 100,000 persons each year. A particulate mucosal vaccine strategy combining antigens and immune stimulator molecules from *Vibrio cholerae* to overcome this problem is described. Proteoliposomes extracted from *V. cholerae* O1 were transformed into cochleates (AFCo2, Adjuvant Finlay cochleate 2) through a calcium inducible rotary dialysis method. Light microscopy was carried out and tubules of 16.25 ± 4.57 μm in length were observed. Western blots were performed to verify the immunochemical properties of the main AFCo2 incorporated antigens, revealing full recognition of the outer membrane protein U (OmpU), lipopolysaccharide (LPS), and mannose-sensitive hemagglutinin (MSHA) antigens. AFCo2 were administered by the intranasal route using a two or three dose schedule and the immune response against *V. cholerae* antigens was assessed. Three AFCo2 doses were required to induce significant ($p < 0.05$), antigen specific IgA in saliva (1.34 ± 0.135) and feces (0.60 ± 0.089). While, two or three doses of AFCo2 or proteoliposomes induce similar specific IgG and vibriocidal activity responses in sera. These results show for the first time that AFCo2 can be obtained from *V. cholerae* O1 proteoliposomes and have the potential to protect against the pathogen when administered intranasally.

1. 1 introduction

Cochleates are phospholipid precipitates derived from the interaction of anionic lipid vesicles with divalent cations such as calcium. They have a tubular shape with a structure consisting of a continuous, solid, lipid bilayer sheet, rolled into a spiral, with a hydrophobic internal space. These structures have been used to deliver protein, peptides, and DNA for vaccine candidate applications via both the oral or nasal routes [1]. A novel and proprietary strategy developed at the Finlay Institute [2] employs proteoliposomes (PL) from bacteria as a source of lipids, pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), and traces of DNA, as well as antigens to induce calcium–cochleate formation using a dialysis rotary process or cross-flow ultrafiltration [2]. The Adjuvant Finlay Cochleate 1 (AFCo1) derived from *Neisseria meningitidis* B PL is more stable and immunogenic than PL administered intranasally [3] AFCo1 has a remarkable adjuvant effect not only enhancing the immune response against its own PL related antigens, but also to other non-related antigens such as ovalbumin or *Leishmania* antigens [4] incorporated in their structure during the manufacturing process.

Cholera is an enteric infection which has drastically affected the global population [5,6]. *Vibrio cholerae* serogroups O1 and O139 are the causative agents of the disease. Serogroup O1 is divided into Classic and El Tor biotypes. The latter has the greatest worldwide distribution and is principally responsible for the last cholera pandemic [7]. A strong and long-lasting protective immunity has been found in convalescents and it is believed that the anti-bacterial response is elicited principally against LPS [8].

Various types of oral cholera vaccines have been commercially developed. The first is based on inactivated whole *V. cholerae* cells supplemented with purified recombinant cholera toxin B-subunit (rCTB) (Dukoral™, licensed by SBL Vaccine, Sweden) [9,10]. The second is a live-attenuated vaccine (Orochol™, Berna Biotech, Switzerland) [11], which has not been produced since 2004 [12]. The third is a vaccine candidate developed in Vietnam; it is a variant of the first without rCTB [13]. Recently, the addition of an O139 serogroup inactivated strain into a reformulated bivalent formulation (*V. cholerae* O1 and O139) has been tested in clinical trials [14].

* Corresponding author. Address: Immunology Department, Bacterial Vaccines Division of Finlay Institute, Ave 17, 198 Street, P.O. Box 16017, Havana City, Cuba. Fax: +53 7 208 6075.
E-mail address: racevedo@finlay.edu.cu (R. Acevedo).
1046-2023/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2009.03.027

Other candidates based on live-attenuated oral cholera vaccines are being evaluated, such as the Peru-15 and Cuban 638 strains [15,16] as well as a whole cell inactivated tablet formulation at preclinical trial stage to overcome administration problems related to price, stability and doses [17]. Nevertheless, the problem with cholera is yet to be resolved [18] and new strategies based on subunit vaccines using immunogenic antigens such as LPS, CT and pilus/porin derived proteins [19–21] have been tested [22,23].

Recently, we prepared a detergent extract of outer membrane components of *V. cholerae* [24] and demonstrated that phospholipids, LPS, and protein antigens (such as MSHA pilus and OmpU porin) are assembled into a proteoliposome-like structure [25], which is capable of inducing vibriocidal activity when administered by the nasal route. In the present paper, we expand the cochleate formation strategy to other microorganisms such as *V. cholerae* to develop a novel mucosal formulation against this pathogen. First, a dialysis process against calcium was used to transform cholera PL (PLc) into cochleates. The major components of PLc (LPS, proteins, and phospholipids) were spontaneously reassembled into a tubular microstructure observed using light microscopy and termed the Adjuvant Finlay Cochleate 2 (AFCo2). The adjuvant effect of AFCo2 was evaluated using a three (0, 7, 14) or two (0, 28) dose intranasal schedule on BALB/c mice and a 638 attenuated vaccine candidate (638) was used as a positive control [26]. Mucosal and systemic immune responses were evaluated based on antibodies secreted in saliva, feces, and sera. Finally, vibriocidal sera titers were also evaluated, as it is believed that these could be correlated with protection, because vibriocidal activity is the best marker of *V. cholerae* immune responses that correlates with protection in humans [27]. Notably, AFCo2 was more immunogenic than PLc at mucosal level and induced immune responses comparable with the 638 positive control.

2. Materials and methods

2.1. Bacterial strains and culture conditions

V. cholerae C7258, El Tor Ogawa [28] was used to obtain the PLc and *V. cholerae* VC12, Classical Ogawa was used as an indicator strain in vibriocidal tests. Bacteria were cultured in peptone from meat (MERCK) 3 g/L, tryptone (MERCK, Germany) 17 g/L, K_2HPO_4 (MERCK) 2.5 g/L, and $NaCL_2$ (MERCK) 5 g/L, pH 7.2 for 6 h at 37 °C with rotational agitation. Lyophilized 638 strain (5003 Lot) prepared at the Finlay Institute was used as a positive control in immunogenicity assays.

2.2. Extraction and characterization of *V. cholerae* proteoliposomes

The PLc were obtained from the *V. cholerae* C7258 strain using a detergent protocol of extraction, reported by Pérez et al. [25]. The structural characterization was carried out using electron microscopy and photon correlation spectroscopy with zeta potential analysis. The composition was evaluated using Lowry/SDS–PAGE for proteins, Fiske–Subarrow for phospholipids and Western blot for LPS and the main antigens as described in Sections 2.4–2.8.

2.3. Dialysis rotary method for producing cochleates

PLc were resuspended and adjusted to 1 mg/mL in a buffer containing 30 mmol/L tris(hydroxymethyl)aminomethane (Tris), 3 mmol/L ethylenediaminetetra-acetic acid (EDTA), and 1.5% (w/v) sodium deoxycholate (DOC). AFCo2 formation was performed by detergent elimination and Ca^{2+} incorporation using a rotary dialysis method against wash buffer containing 30 mmol/L Tris, 100 mmol/L NaCl, and 5 mmol/L $CaCl_2$ at pH 7.4. Five washes were carried out at 2 hourly intervals. Organoleptic properties were observed during the process, paying special attention to changes in color and appearance of the suspension. AFCo2 was centrifuged twice at 3000*g* for 10 min to remove non-incorporated components. Incorporation efficiency of LPS, proteins, and phospholipids into AFCo2 was estimated on the basis of the amount of these components (Section 2.6) in the resuspended pellets.

2.4. Photon correlation spectroscopy (PCS) and zeta surface potential

Analysis was carried out using a Zetasizer 3000HS (Malvern Instruments, Malvern, UK). Vesicle size experiments were achieved using a solid-state laser as the light source. The measurements were carried out at a scattering angle of 90°. The correlation functions were performed by a Malvern PCS sub-micron particle analyzer and a third-order cumulate fitting to obtain the mean diameter and polydispersity. The real and imaginary refractive index was set at 1.59 and 0.0, respectively. Zeta potential determination was carried out using Zeta limits ranging from −120 to 120 V and measured in the automatic mode. The samples were diluted with 1 mM NaCl to two different protein concentrations 0.05 and 0.15 mg/mL. Ten measurements were carried out for each experiment. Data are expressed as mean size ± standard deviation.

2.5. Microscopic analysis

Morphological examination of PLc nanoparticles was carried out by transmission electron microscopy (TEM) using a ZEISS DSM-962 microscope. The samples were resuspended in water to 0.05 mg/mL (protein), stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar films for observation. The PLc derived structure was observed by light microscopy using an Opton Standard 25 microscope. The sample was resuspended in wash buffer to 0.1 mg/mL and fixed on a slide. In addition, the size of the structures was determined using a gradation scale on the ocular lens [29].

2.6. Protein, lypopolysaccharide, and membrane phospholipids determinations

The concentration of protein in the samples was calculated by the Lowry method using bovine serum albumin (BSA) as the standard [30]. The quantity of LPS was estimated by the use of Western blot-densitometry [31] and the membrane phospholipid concentration was evaluated using the mineralization method of Fiske and Subarrow [32].

2.7. SDS–PAGE

Samples were incubated at 100 °C for 2 min. in the presence of 2 β-mercapto-ethanol (Merck, Germany) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (acrylamide 12.5%) followed by staining with R250 Coomassie Blue [33]. Molecular weight markers (MW, Bio-Rad) were used to determine the molecular size of the components and identify the predominant proteins in each sample using an image processor (ImageMaster VDS, Pharmacia).

2.8. Western blot

A panel of anti-*V. cholerae* O1 monoclonal antibodies (MAb) was used to evaluate the identity of the main antigens on samples for anti-MSHA (2F12F1) [34], anti-Ogawa LPS against O-antigen-polysaccharide region (O-p-LPS) (2B4G5) [35], and anti-OmpU (9H12E6) [36]. The procedure was followed as described by Burnett [37]. Samples were separated by SDS–PAGE (acrylamide

12.5%), transferred to a nitrocellulose membrane and incubated with the MAb as previously described [25]. An anti-mouse peroxidase-conjugated IgG (Sigma Chemical Co., St. Louis, Mo.) and diaminobenzidine as the substrate were used to develop the blot.

2.9. Immunization and sample collection

Female BALB/c mice (6–8 weeks old, CENPALAB, Cuba) were immunized as follows: PLc and AFCo2 formulations were adjusted to 5 mg of protein/mL and each mouse received 20 μL (10 μL per nostril) by the intranasal route without anesthesia. In addition, two control groups were immunized, one received 20 μL of phosphate buffered saline (PBS) as placebo and the other received 10^8 colony forming units (CFU) of 638 [16] as a positive control. Mice were immunized at days 0 and 28 in a two dose schedule or three doses at days 0, 7, and 14. Saliva and feces were collected 7 days after the last dose and processed as follows. Pooled saliva samples were taken by stimulating salivation using an intraperitoneal injection of 50 μL of 0.5% pilocarpine. The samples were inactivated for 15 min at 56 °C and centrifuged at 10,000*g* for 10 min. Three to six pieces of freshly voided feces were collected into 1.5 mL pre-weighed micro-centrifuge tubes and PBS was added (pH 7.2) with protease inhibitors [1 mM phenylmethyl sulfonylfluoride in ethanol, 5 μg/mL of aprotinin, 1 μg/mL of leupeptin, antipain and pepstatin (all from Sigma)] in a ratio of 20 μL per mg of feces. Solid matter was resuspended by extensive vortexing and centrifuged at 10,000*g* for 10 min. and the supernatants were stored at −20 °C. For serum collection, animals were bled by retro-orbital puncture, 14 days after the last immunization, 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 kept following the Canadian Council directions for laboratory animal experiments. All experiments were performed with approval from the Finlay Institute Ethical Committee.

2.10. Determination of antibodies by ELISA

Anti-PLc IgG antibodies in serum samples and anti-PLc IgA antibodies in saliva and feces were measured by direct ELISA using polystyrene 96-well plates (MaxiSorp F96; Nunc, Roskilde, Denmark). Briefly, plates were coated with PLc (100 μL per well) at 5 μg/mL in Na_2CO_3–$NaHCO_3$ buffer (0.1 mol/L, pH 9.6) at 4 °C overnight, and blocked with 1% (w/v) BSA in PBS (0.15 mol/L, pH 7.3, blocking solution) for 1 h at room temperature. Serum samples were diluted 1:100 and saliva and feces 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 μL 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 H_2O_2 in citrate–phosphate buffer, pH 5). The reaction was stopped by adding 50 μL of H_2SO_4 at 2 mol/L and the 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% (v/v) Tween-20. Anti-PLc IgG and anti-PLc IgA antibodies were expressed in optical density units. The means and standard deviation of at least three different experiments are shown.

2.11. Vibriocidal antibody assay

Serum vibriocidal antibodies were determined as described previously [38]. Briefly, 50 μL of two-fold dilutions of decomplemented (heat inactivated) sera in PBS were mixed with 50 μL of a *V. cholerae* VC12 (serotype Ogawa) suspension containing 10^8 CFU/mL in PBS supplemented with 2% (w/v) guinea pig complement and placed in a 96-well microtiter plate. The mixture was incubated for 1 h at 37 °C and subsequently supplemented with 100 μL of Brain Heart Infusion Broth containing 2% (w/v) dextrose and 0.02% (w/v) bromocresol purple. The plates were incubated at 37 °C for 3 h. Bacterial growth was determined by a color change of the medium, which indicated bacterial dextrose consumption. The serum dilution in the first column of the plate was 1:20 and in the last 1:10240. The vibriocidal antibody titer was calculated as the inverse of the highest dilution of serum causing complete inhibition of bacterial growth (no change of color in the medium). The results were expressed as $\log_{10}$ titer.

2.12. Statistical methods

Statistical analysis of the data was carried out using ANOVA or the non-parametric Kruskal–Wallis test. Post test multiple comparison was performed with Graph Pad Prism 4 software (CA, USA).

3. Results

3.1. V. cholerae proteoliposome extraction and Characterization

SDS detergent was used to extract the PLc from a virulent strain of C7258, *V. cholerae* O1, El Tor Ogawa and physico-chemical characterization was carried out as previously reported [24,25]. The amount of LPS was determined using densitometric analysis of a Western blot against a standard curve of LPS from *V. cholerae* O1. The amount was calculated as 0.28 ± 0.06 mg of LPS per 1 mg of protein. Total protein was calculated using a Lowry assay and total phospholipid was quantified as 0.62 ± 0.12 mg per 1 mg of total protein using the mineralization method of Fiske and Subarrow. SDS PAGE protein analysis shows a profile with several proteins and a major band at ∼38 kDa (Fig. 1 lane 2). The average particle size of PLc, 160.7 ± 1.6 nm nm, was determined using PCS with a Zetasizer 3000HS (Malvern Instruments, Malvern, UK). The negatively charged surface of the vesicles was also estimated as −23.8 ± 1.21 mV. TEM micrograph confirmed the nanosize and vesicle shape of the PLc, which appear to be composed of a single lamellar structure (Fig. 2).

3.2. AFCo2 formation strategy and characterization

PLc were transformed into AFCo2 using the dialysis rotary method, which was characterized by a slow change of the organo-

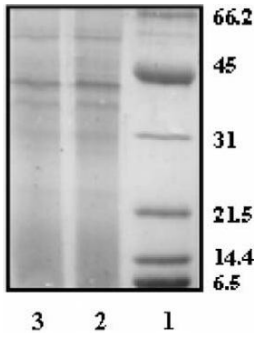

Fig. 1. Protein profile of proteoliposome (PLc) and cochleate (AFCo2) structures from *V. cholerae*. Separation was achieved by SDS–PAGE (acrylamide 12.5%) and visualized with R250 Coomasie Blue stain. The samples were incubated at 100 °C for 2 min. in the presence of 2 β-mercapto-ethanol (Merck): Lane 1: MW, Lane 2: PLc (10 μg), Lane 3: AFCo2 (10 μg). OmpU is the major protein observed at ∼38 kDa in Lanes 2 and 3.

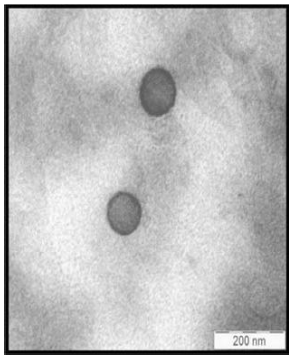

Fig. 2. Transmission Electron Microscopy of *V. cholerae* proteoliposomes. The micrograph was carried out with a ZEISS DSM-962 at 10 kV. Two vesicles are shown at 10,000× magnification.

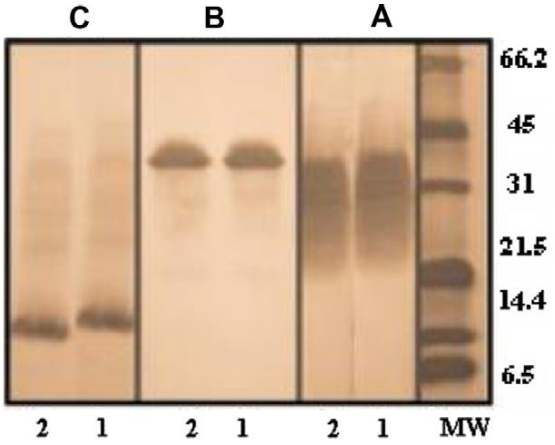

Fig. 4. Immunochemical analysis of the main antigens in AFCo2 after PLc transformation, carried out by Western blot analysis. Lanes 1 represent blotted PLc (5 μg) and lanes 2 the AFCo2 (5 μg). MW shows biotinylated molecular weight markers. Each panel was treated with different MAbs. (A): MAb 2B4G5 (anti O-p-LPS Ogawa), (B): MAb 9H12E6 (anti-OmpU) and (C): MAb 2F12F1 (anti-MSHA) and reveal LPS at ∼30 kDa (A), OmpU at ∼38 kDa (B) and MSHA at ∼17 kDa (C).

leptic properties of the colorless solution of PLc. Initially, it was a white milky precipitate consisting of large particles. Tubular microstructures were observed by light microscopy (Fig. 3A) with an average size of 16.25 ± 4.57 μm (Fig. 3B). The efficiency of major components to be incorporated during PLc transformation into the AFCo2 was calculated. Protein, LPS, and phospholipids were incorporated with an efficiency of 95.3, 92.5, and 93.7%, respectively. The protein profiles of PLc and AFCo2 analyzed by SDS/PAGE are shown in Fig. 1 lanes 2 and 3, respectively. Densitometric analysis showed no significant differences between the areas under the curve representing the major bands in this figure (data not shown). The identity of the main antigens after PLc transformation was evaluated using a Western blot with MAbs against MSHA, LPS, or OmpU porin. Each of them was positively identified in AFCo2 and PLc (Fig. 4).

3.3. Mucosal immune response induced by intranasal AFCo2

BALB/c mice were immunized via the intranasal route using a two (days 0 and 28) or three dose schedule (days 0, 7, and 14). The anti-PLc IgA response was measured by ELISA using PLc as the coating antigen. Three doses of AFCo2 showed significant enhancement of anti-PLc IgA in saliva ($p < 0.01$) and feces ($p < 0.05$) compared with those immunized with PLc or 638 (Fig. 5A and B). Two doses of AFCo2 induced no significant differences between anti-PLc IgA responses in saliva ($p > 0.01$) and feces ($p > 0.05$) compared with PLc and they were significantly ($p < 0.05$) lower than those treated with three doses. Moreover, the antibody titers in saliva and feces induced by AFCo2 or PLc at any schedule were significant ($p < 0.05$) when compared with that induced by three doses of 638. Importantly, when responses in saliva and feces of both schedules were compared (Fig. 5 A and B), three doses of AFCo2 induced a specific IgA mucosal response comparable only with two doses of 638. Overall, AFCo2 induced the highest anti-PLc IgA with three doses.

3.4. Systemic immune response induced by intranasal AFCo2

The systemic specific IgG response and vibriocidal activity in sera was assessed. Table 1 shows strong systemic anti-PLc IgG responses with no significant differences between the experimental groups. Furthermore, both AFCo2 treatment schedules show vibriocidal titers as high as the PLc and the two dose 638 control group, but the 638 immunized mice with a three dose schedule elicited significantly ($p < 0.01$) lower IgG responses than AFCo2 or PLc. Overall, these data show that AFCo2 induce a similar systemic response when immunized by either schedule.

4. Discussion

Development of mucosal vaccine strategies against bacterial infections based on particulate non-living delivery systems [57] are one of the most important research goals at the Immunology Department of Finlay Institute, not only because they represent a

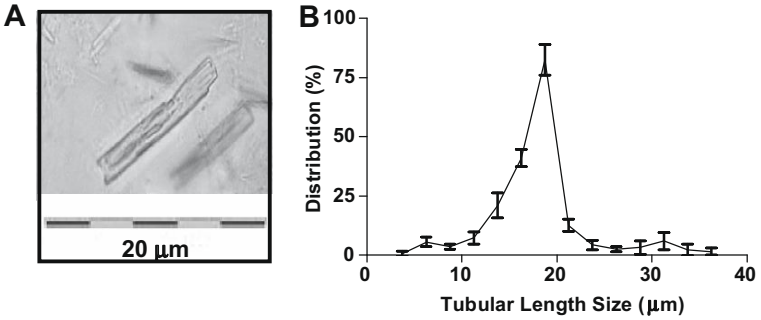

Fig. 3. Light Microscopy and size analysis of *V. cholerae* cochleates (AFCo2). (A) AFCo2 micrograph carried out with an Opton Standard 25 microscope 400×. (B) Distribution percentage (82.6%) of AFCo2 with length size of 16.25 ± 4.57 μm was obtained from three processes and measured by a graduated scale on the ocular lens of the Opton light microscope.

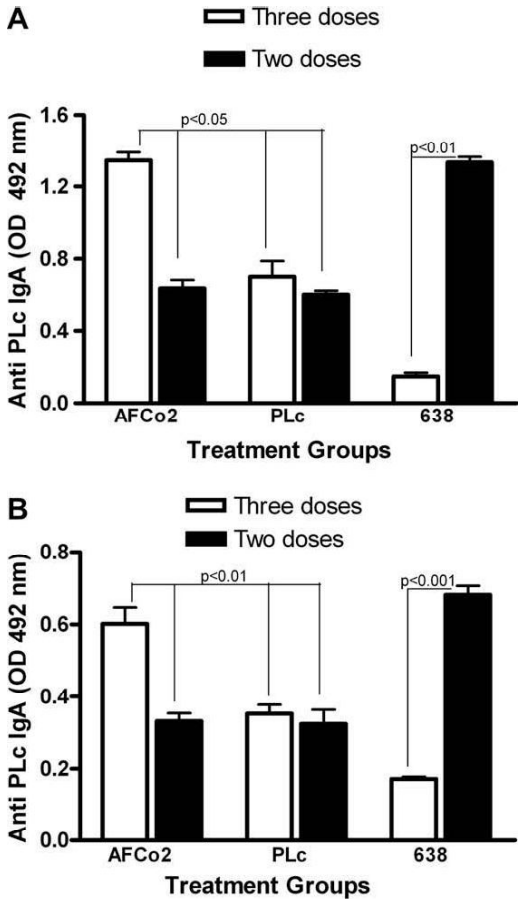

Fig. 5. Specific IgA antibody response to *V. cholerae* proteoliposome (PLc) antigens in mice saliva (A) and feces (B). Hundred microgram of AFCo2 and PLc (50 μg each nostril), 10^8 UFC of 638 and placebo were administered to mice groups ($n = 5$) by the intranasal route (10 μL per nostril) using either a three or two dose schedule. Seven days after the last immunization, saliva and feces were collected and diluted 1:2. Results are expressed as a mean of optical density units (OD) ± standard deviation. The placebo effect was subtracted for statistical analysis. Dunn's multiple comparison test (saliva) or Tukey test (feces) were used to analyze the data of three experiments.

Table 1
Systemic immune response induced by different immunization schedules on BALB/c mice.

Treatment groups	IgG[a]		Vibriocidal activity[b]	
	Two doses	Three doses	Two doses	Three doses
PLc	0.626 ± 0.18	0.633 ± 0.09	2.054 ± 0.41	2.024 ± 0.29
AFCo2	0.661 ± 0.14	0.557 ± 0.12	2.271 ± 0.33	2.294 ± 0.42
638	0.556 ± 0.22	0.125 ± 0.09**	2.415 ± 0.37	1.512 ± 0.40*

Mice were immunized intranasally with 100 μg of AFCo2 and PLc or 108 UFC of 638. Tukey multiple comparison test was used to analyze the data of three experiments ($n = 5$).

[a] Results are expressed as mean of optical density units (DO) ± standard deviation.

[b] Results are expressed as mean of $\log_{10}$ titer ± standard deviation.

* Indicates values significantly lower than the three dose of PLc or AFCo2 ($p < 0.05$).

** Indicates values significantly lower than the three dose of PLc or AFCo2 ($p < 0.01$).

pain free alternative, but also because they provide the potential for both mucosal and systemic protection. Previously, we have demonstrated the benefits of using proteoliposomes prepared from *V. cholerae* (PLc) [25] in the nasal induction of serum vibriocidal activity and other authors have also found that native outer membrane vesicles from cholera are protective [39]. The aim of this work is to demonstrate that PLc can be transformed into a cochleate structure (AFCo2), which can then be used successfully as an adjuvant to increase the mucosal and systemic immune responses against *V. cholerae* antigens to intranasally immunized mice. For that reason PLc were extracted from C7258 wild type strain and we evaluated the potential for using them to obtain a cochleate structure. Structural studies using correlation spectroscopy and TEM revealed the PLc to have a nanometric size (160.7 ± 1.6 nm) and a negative charged surface of −23.8 ± 1.21 mV supporting the possibility that calcium interacts with the negative surface components of PLc as previously reported [40]. Furthermore, TEM micrographs confirm the vesicle shape of the PLc and point at the possibility of them being unilamellar vesicles, although further characterization is required to establish this. As previously reported by us [2], vesicle structures from microorganisms can be transformed into cochleates by rotary dialysis on a laboratory scale or by cross-flow ultrafiltration for industrial/pilot scale [41]. The principle of these processes is that calcium interacts with negatively charged proteins, phospholipids, and others structures from proteoliposomes to form cochleates. In this study, rotary dialysis was successfully used to transform the PLc into AFCo2. A tubular microparticle of 16.25 ± 4.57 μm of length was obtained as previously described [42]. The dialysis process was characterized by a slow change in the organoleptic properties of a colorless solution of PLc resuspended in a suitable detergent in a dialysis bag to a white milky precipitate of large particles appearing after the first wash. However, further studies will be needed to characterize the supramolecular structure of AFCo2.

One of the challenges of this formation process was to efficiently incorporate the principal components of PLc in the cochleate structure. Phospholipids and LPS from PLc represent 50–60 and 25–30%, respectively of total protein. Incorporation of PLc major components in AFCo2 was subsequently analyzed and we found that more than 90% of LPS, phospholipids, and proteins efficiently remain in the structure after centrifugation and elimination of un-entrapped material.

The identity and integrity of PLc and AFCo2 main antigens was evaluated using SDS–PAGE and Western blots. SDS–PAGE profiles of the samples show multiple bands and a major band at ~38 kDa that corresponds with OmpU porin showing no integrity damage on the protein profile after dialysis. However, in order to determine whether the immunochemical properties of the main antigens in AFCo2 would have been affected during PLc transformation by the action of calcium, detergent or any other substance used in the washing buffer, Western blot analysis using anti-MSHA, anti-LPS or anti-OmpU MAbs was carried out. The results reveal full recognition of the OmpU, the LPS, and the MSHA antigens in the PLc and AFCo2. This is very important because OmpU has been reported as one of the major *V. cholerae* outer membrane proteins [43] and considered a potential adherence factor of this microorganism [44]. LPS induces protective immune responses in humans and animals [45,46] and has been widely accepted as a protective immunogen for *V. cholerae* vaccine development [47,48] as well as considered an important immunopotentiator molecule inducing dendritic cell activation through toll like receptor 4 [49]. Finally, MSHA pilus protein has been associated with protective immune responses suggesting that it may play an important role in intestinal colonization [50,19]. Nevertheless, *in vivo* evaluation of the immunological properties of AFCo2 would be the main task to demonstrate the adjuvant potential of transformation on PLc antigens. Mucosal vaccination is considered a subject of great interest due to advantages above the parenteral route of immunization. One effective way to induce mucosal immunity using AFCo1 is to administer it by intranasal immunization [2]. Some authors have used this route to immunize

against intestinal pathogens in order to avoid the adverse gastrointestinal conditions for stability and absorption of vaccine candidates [51]. For that reason the intranasal route was used to immunize mice using two schedules. Results showed no significant differences between PLc and AFCo2 responses induced at mucosal or systemic level with the two doses schedule. Confirming that PLc transformation into AFCo2 did not affect antigen immunogenicity and neither has adjuvant effect on cholera antigens with this regime. Notably, mucosal specific IgA responses in saliva and feces induced by PLc are reported for the first time by us. Nevertheless, when we compared AFCo2 with 638 immunized mice with two doses, the response observed by the control group was higher than the rest of the groups ($p < 0.01$). It was interesting because we have not had any reports about mucosal immune responses induced by 638 when intranasally administered. As the best schedule using AFCo1 by the intranasal route was three doses, 7 days apart [2], we decided to assess this schedule with AFCo2. Thus, mice were immunized with AFCo2, PLc, or 638 using this protocol (0, 7, 14) and systemic responses were evaluated. Once more, there were no significant differences between PLc and AFCo2 ($p < 0.05$) for specific IgG and vibriocidal responses. However, the mucosal response revealed that AFCo2 induced higher specific IgA responses in saliva and feces than PLc and also when compared with itself using a 0–28 schedule. This result could be due to better antigenic presentation in nasal tissues with the AFCo2, and also antigen stability preservation owing to transformation, that could be essential for a successful controlled delivery platform. Some authors have reported that particulate structures exceeding 5 μm are better retained in inductive sites and stimulate local mucosal immune responses while smaller antigens escaping to the peripheral lymphatics will induce systemic immune responses [52,53]. That is why transformation of a nanoparticle like PLc into a AFCo2 microstructure could improve the mucosal immune response. In addition, microparticles > 15 μm are considered optimal for nasal application [54]. The importance of delivery systems, their size, shape and other structural features could be of vital importance in the design of mucosal adjuvants and vaccines, taking into account that tolerance can be induced by this route, as well as the oral [57]. We then evaluated mucosal responses induced by AFCo2 and found them to only be comparable with that elicited in mice using two doses of 638. In general, 638 was not effective in inducing systemic and mucosal immunity in immunized mice with a three dose schedule. Considering that 638 is a live-attenuated vaccine, the kinetics to induce immunity are very different from that for AFCo2 or PLc. Therefore, Silva et al. [26] used a two dose schedule. Moreover, the adjuvant effect of PLc transformation into AFCo2 is demonstrated at mucosal level using the three dose schedule. O'Hagan [55] recommends three doses to evaluate adjuvant potential on vaccine formulations administered by the intranasal route. Our group also found this schedule to be best with AFCo1 [2], moreover recent clinical trials using a gel vaccine candidate against *Pseudomonas aeruginosa* also demonstrated that a three dose schedule was better than two doses to improve immune responses elicited against this antigen [56]. Overall, AFCo2 immunized with a three dose schedule was the only treatment with comparable immune responses to the 638 positive control (0, 28). Some authors [58,59] have also suggested that the intranasal route is not preferable for inducing mucosal immunity in the gastrointestinal tract and oral immunization will not be effective in inducing a good immune response at systemic level. However, our results demonstrate that the adjuvant potential of the formulations, dose and schedule could be determinant to overturn this idea. In addition, other groups have obtained good immune responses in intestinal samples [60] using the intranasal route of immunization or systemic responses using the intragastric route [61]. In any case, the mechanism of action behind all this evidence is not clear and further work is needed to improve our knowledge on mucosal immunology and vaccine design.

5. Conclusions

A proteoliposome-derived cochleate (AFCo2) from *V. cholerae* O1 was obtained using a rotary dialysis method. This result confirms that the methodology for AFCo1 production, at least at laboratory level, could be used to also obtain AFCo2 from PLc. The tubular microparticle carries important immunopotentiators and immunogenic molecules that were able to induce higher systemic and mucosal specific immune responses than PLc when administered intranasally to mice. This demonstrates the adjuvant effect of PLc transformation into AFCo2 at mucosal level using a three dose schedule. Finally, we found that the three dose regime was most advantageous for our future studies to obtain a cochleate multiple formulation against enteric pathogens.

Acknowledgments

This research was supported by the Finlay Institute. We thank to Dr. Gustavo Falero for kindly providing access to monoclonal antibodies against *V. cholerae* antigens, Domingo González for providing the 638 vaccine candidate and Dr. Arturo Talavera for useful suggestions.

References

[1] S. Gould-Forgerite, M.T. Kheiri, F. Zhang, Z. Wang, A.J. Scolpino, E. Feketeova, et al., Adv. Drug Deliv. Rev. 32 (1998) 273–287.
[2] O. Pérez, G. Bracho, M. Lastre, G. Sierra, C. Campa, N. Mora et al., Método de obtención de estructuras cocleares, composiciones vacunales y adyuvantes basados en estructuras cocleares, OCPI23313 (Cuban Office for Intellectual Property), 2008.
[3] O. Pérez, M. Lastre, O. Cabrera, J. del Campo, G. Bracho, M. Cuello, et al., Scand. J. Immunol. 66 (2007) 271–277.
[4] J. del Campo, M. Lastre, G. Bracho, T. Rodríguez, D. Gil, C. Zayas, et al., Vaccine 24 (S2) (2006) 50–51.
[5] E.D. Mintz, R.V. Tauxe, M.M. Levine, The global resurgence of cholera, in: N. Noah, M. O'Mahony (Eds.), Communicable Disease: Epidemiology and Control, John Wiley & Sons, Chichester, England, 1998, pp. 63–104.
[6] C.O. Tacket, M.B. Cohen, S.S. Wasserman, G. Losonsky, S. Livio, K. Kotloff, et al., Infect. Immun. 67 (1999) 6341–6345.
[7] D. Sack, R.B. Sack, G.B. Nair, A.K. Nair, A.K. Siddique, Lancet 363 (2004) 223–233.
[8] M.M. Levine, J.B. Kaper, Live oral cholera vaccine: from principle to product, Bull. Inst. Pasteur 93 (1995) 243–253.
[9] J.L. Sanchez, A.F. Trofa, D.N. Taylor, R.A. Kuschner, R.F. DeFraites, S.C. Craig, et al., J. Infect. Dis. 167 (6) (1993) 1446–1449.
[10] J.L. Sanchez, B. Vasquez, R.E. Begue, R. Meza, G. Castellares, C. Cabezas, et al., Lancet 344 (8932) (1994) 1273–1276.
[11] E.E. Richie, N.H. Punjabi, Y.Y. Sidharta, K.K. Peetosutan, M.M. Sukandar, S.S. Wasserman, et al., Vaccine 18 (2000) 2399–2410.
[12] J.F. Viret, G. Dietrich, D. Favre, Vaccine 22 (2004) 2457–2469.
[13] D.D. Trach, P.D. Cam, N.T. Ke, M.R. Rao, D. Dinh, P.V. Hang, et al., Bull. WHO 80 (2002) 2–8.
[14] D. Mahalanabis, A.L. Lopez, D. Sur, J. Deen, B. Manna, S. Kanungo, et al., PLoS ONE 3 (6) (2008) e2323.
[15] F.M. Qadri, I. Chowdhury, S.M. Faruque, M.A. Salam, T. Ahmed, Y.A. Begum, et al., Vaccine 25 (2007) 231–238.
[16] L. García, J.M. Díaz, H. García, B.L. Rodríguez, R. Fernández, G. Año, et al., Infect. Immun. 73 (5) (2005) 3018–3024.
[17] A. Talavera, G. Año, Y. Pino, J. Castaño, E. Uribarri, L. Riverón, et al., Vaccine 24 (2006) 3381–3387.
[18] C.L. Chaignat, Exp. Rev. Vaccines 7 (4) (2008) 403–405.
[19] J. Osek, A.M. Svennerholm, J. Holmgren, Infect. Immun. 60 (1992) 4961–4964.
[20] S.N. Chatterjee, K. Chaudhuri, Biochim. Biophys. Acta. 1762 (2006) 1–16.
[21] V. Sperandio, J.A. Giron, W.D. Silveira, J.B. Kaper, Infect. Immun. 63 (1995) 4433–4438.
[22] R.K. Gupta, S.C. Szu, R.A. Finkelstein, J.B. Robbins, Infect. Immun. 60 (1992) 3201–3208.
[23] O. Cabrera, M.E. Martinez, M. Cuello, C.R. Soto, T. Valmaseda, B. Cedre, et al., Vaccine 24 (S2) (2006) 74–75.
[24] J.L. Pérez, Y. González, G. Año, B. Cedré, T. Valmaseda, M. Álvarez, et al., VacciMonitor 15 (1) (2006) 1–7.
[25] J.L. Pérez, R. Acevedo, Y. Fernández, B. Cedré, G. Año, L. González, et al., Vaccine (2008), doi:10.1016/j.vaccine.2008.10.052.
[26] A.J. Silva, A. Mohan, J.A. Benitez, Vaccine 21 (2003) 4715–4721.
[27] W.H. Mosley, W.M. McCormack, A. Ahmed, A.K.M. Chowdhury, R.K. Barui, Bull. World Health Organ. 40 (2003) 187–197.

[28] C.C. Hase, L.S. Thai, M.B. Finkelstein, V.L. Mar, W.N. Burnette, H.R. Kaslow, et al., Infect. Immun. 62 (1994) 3051–3055.
[29] D. Gil, G. Bracho, C. Zayas, J. del Campo, R. Acevedo, A. Toledo, et al., Vaccine 24 (S2) (2006) 92–93.
[30] O. Lowry, H. Rosebrough, A. Farr, R. Randall, A protein measurement with the folin-phenol reagent, Biol. Chem. 193 (1951) 265–275.
[31] J.L. Pérez, Y. Fernández, Y. Aranguren, M. Alvarez, R. Acevedo, O. Pérez, .L. García, VacciMonitor 16 (1) (2007) 17–22.
[32] C.N. Fiske, Y. Subarrow, J. Biol. Chem. 66 (1925) 375.
[33] N.K. Laemmli, Nature 227 (1970) 680–685.
[34] G. Falero, B.L. Rodriguez, T. Valmaseda, M.E. Perez, J.L. Perez, R. Fando, et al., Hybridoma 17 (1) (1998) 63–67.
[35] G. Año, H. Garcia, T. Valmaceda, B. Cedre, Y. Pino, O. Ancheta, et al., Biotechnol. Apl. 20 (1) (2003) 9–13.
[36] G. Falero, I. Rodríguez, E. Susarte, O. Otero, R. Fando, N. Nuñez, et al., Rev. CNIC Cienc. Biol. 13 (3) (2007) 15–19.
[37] W.N. Burnette, Anal. Biochem. 112 (1980) 192–200.
[38] J. Benitez, L. García, A. Silva, H. Garcia, R. Fando, B. Cedré, et al., Infect. Immun. 67 (1999) 539–545.
[39] S. Schild, E.J. Nelson, A. Camilli, Infect. Immun. 76 (10) (2008) 4554–4563.
[40] G. Bracho, M. Lastre, J. del Campo, C. Zayas, D. González, D. Gil, et al., Vaccine 24 (S2) (2006) 30–31.
[41] C. Zayas, G. Bracho, M. Lastre, D. González, D. Gil, R. Acevedo, et al., Vaccine 24 (S2) (2006) 94–95.
[42] O. Pérez, G. Bracho, M. Lastre, G. Sierra, C. Campa, N. Mora et al., Method of obtaining cochlear structures, vaccine compositions, adjuvants and intermediates thereof, EP 1602360 and US 20060134134 A1, 2005.
[43] V.L. Miller, J.J. Mekalanos, J. Bacteriol. 170 (1988) 2575–2583.
[44] V. Sperandio, J.A. Giron, W.D. Silveira, J.B. Kaper, Infect. Immun. 63 (1995) 4433–4438.
[45] F.M. Apter, P. Michetti, L.S. Winner, J.A. Mack, J.J. Mekalanos, M.R. Neutra, Infect. Immun. 61 (1993) 5279–5285.
[46] A.S. Benenson, W.H. Mosley, M. Fahimuddin, R.O. Oseasohn, Bull. World Health Organ. 38 (3) (1968) 359–372.
[47] L. Winner, J. Mack, R. Weltzin, J.J. Mekalanos, J.P. Kraehenbuhl, M.R. Neutra, Infect. Immun. 59 (1968) 977–982.
[48] A.M. Svennerholm, J. Holmgren, Infect. Immun. 13 (1976) 735–740.
[49] S.M. Zughaier, S.M. Zimmer, A. Datta, R.W. Carlson, D.S. Stephens, Infect. Immun. 73 (5) (2005) 2940–2950.
[50] T.M. Finn, J. Reiser, R. Germanier, S.J. Cryz, Infect. Immun. 55 (4) (1987) 942–946.
[51] L.F. Fries, A.D. Montemarano, C.P. Mallett, D.N. Taylor, T.L. Hale, G.H. Lowell, Infect. Immun. 69 (7) (2001) 4545–4553.
[52] S. Espuelas, J.M. Irache, C. Gamazo, Inmunología 24 (2) (2005) 208–223.
[53] Y. Matsunaga, Y. Wakatsuki, Y. Tabata, H. Kawasaki, T. Usui, M. Yoshida, et al., Vaccine 19 (4–5) (2000) 579–588.
[54] L. David, Mucosal Adjuvants. St. George's Vaccine Institute, UK, 2008. Available from: <www.who.int/vaccine_research/about/gvrf/Lewis.pdf>.
[55] D.T. O'Hagan, Vaccine Adjuvants. Preparation Methods and Research Protocols, Humana Press, Inc., Totowa, NJ, 2000.
[56] U. Baumann, K. Göcke, B. Gewecke, J. Freihorst, B.U. Von Specht, Respir. Res. 8 (1) (2007) 57.
[57] J. Mann, R. Acevedo, J. del Campo, O. Perez, V. Ferro, Expert Rev. Vaccines 8 (1) (2009).
[58] F.E. Johansen, E.S. Baekkevold, H.S. Carlsen, I.N. Farstad, D. Soler, P. Brandtzaeg, Blood 106 (2) (2005) 593–600.
[59] P. Brandtzaeg, Vaccine 25 (30) (2007) 5467–5484.
[60] M.E. Drago-Serrano, H.A. Manjarréz, S. Gavilanes, T. del Rosario, Rev. Latinoam. Microbiol. 46 (1–2) (2004) 17–23.
[61] C.P. Mallett, T.L. Hale, R.W. Kaminski, T. Larsen, N. Orr, D. Cohen, et al., Infect. Immun. 63 (6) (1995) 2382–2386.