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A proteoliposome based formulation administered by the nasal route produces vibriocidal antibodies against El Tor Ogawa *Vibrio cholerae* O1 in BALB/c mice

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ABSTRACT

A vaccine candidate against the enteric pathogen *Vibrio cholerae* was developed based on a proteoliposome (PL) formulation using a wild type strain C7258, *V. cholerae* O1, El Tor Ogawa as part of strategy to develop a combined formulation against enteric diseases preventable by the stimulation of the mucosal immune system. A detergent extraction method was applied to obtain the PL. Scanning electron microscopy and molecular exclusion chromatography showed the presence of two PL populations. Photon correlation spectroscopy studies were then carried out to evaluate the size (169.27 ± 3.85 nm), polydispersity (0.410) and zeta potential (-23.28 ± 1.21 mV) of the PL. SDS-PAGE and Western blot analysis revealed the presence of lipopolysaccharide (LPS), mannose-sensitive haemagglutinin (MSHA) and a range of outer membrane proteins, including OmpU. BALB/c mice were immunized intranasally with two doses of PL containing 25 μg of LPS each 28 days apart. The mice showed high anti-LPS IgG titres (3.36 ± 0.235) and vibriocidal antibodies (3.70 ± 0.23) after two weeks from last dose. These results show for the first time that PL can be obtained from *V. cholerae* O1 and when administer by intranasal route has the potential to protect against this pathogen.

1. Introduction

Cholera is an enteric infection which has drastically affected the global population [1,2] and is still a serious health problem [3]. *Vibrio cholerae*, serogroups O1 and O139 are the causative agents of the disease. Serogroup O1 is divided into two biotypes: classic and El Tor. The latter is the most worldwide distributed and is principally responsible for the last cholera pandemic [3]. 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 the cell wall component lipopolysaccharide (LPS) [4].

Currently, two types of oral *V. cholerae* vaccines are available. One is based on inactivated whole cells and the other is a live attenuated vaccine. Both have been shown to be safe, immunogenic and efficacious [5,6]. These two vaccines have been licensed in some countries and are mainly used by travellers from industrialized countries to endemic areas, but they are now under consideration for their use in developing countries as an additional control measure against *V. cholerae*. Several countries have already performed mass vaccination on high-risk populations [7].

The inactivated vaccine (Dukoral™, licensed by SBL Vaccine, Sweden) consists of four batches of heat- or formalin-killed whole-cell *V. cholerae* O1, representing both serotypes (Inaba and Ogawa) and both biotypes (Classical and El Tor) supplemented with purified recombinant cholera toxin B-subunit (rCTB). This vaccine is given orally with buffer to neutralize stomach acidity and in field trials in USA and in field trials in USA and Peru was found to confer 80–90% protection over six months in all age groups tested following administration of two doses 1–2 weeks apart [7–9]. On the other hand, the attenuated vaccine consists of a live attenuated genetically modified *V. cholerae* O1 Inaba strain (CVD103-HgR), which has been engineered to produce CTB but not the A subunit of CT. The vaccine (Orochol™, Berna Biotech, Switzerland) is given orally along with buffer to neutralize stomach acidity. This vaccine is available in two formulations, either a low dose formulation for non-endemic countries or a 10-fold higher dose formulation for cholera-endemic developing countries. Efficacy in adult volunteers in the USA was found to be about 80% against all diarrhoea and 90% against severe diarrhoea following challenge with *V. cholerae* O1 (of either El Tor or Classical biotype) given three months after vaccination. However, in a subsequent large field trial performed in a cholera-endemic area of Indonesia on 67,000 volunteers, the vaccine failed to demonstrate protection, due to the limited numbers of cholera cases recorded during the trial [7,10].

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Recently, a reformulated bivalent (*V. cholerae* O1 and O139) killed whole-cell oral vaccine developed in Viet Nam was assessed for safety and immunogenicity in a cholera-endemic area in India. The vaccine was shown to be safe and immunogenic [11].

Our group has been working on a subunit cholera vaccine candidate derived from detergent extracts of outer membrane components of *V. cholerae* [12]. This method results in proteoliposome (PL) structures, but only if there is correct assembly of the major components; in this case phospholipids, proteins and LPS. To date, there are only few examples of licensed and available PL vaccines. One of them, the Cuban anti-meningococcal vaccine, VA-MENGOC-BC™ [13] is a classic example of a vaccine compound by a PL obtained from the outer membrane of *Neisseria meningitidis* serogroup B. This *N. meningitidis* PL has proved to have potent adjuvant activity when used with other vaccine antigens and not only in its native vesicle form but also in cochleate form [14]. PL are also stable and non-related antigens can be incorporated in them to be used as delivery system [15]. In contrast to liposomes, PL from bacteria contain LPS, proteins and other molecules in their structure known as pathogen associated molecular patterns (PAMPs) with immune potentiator and modulator effects [16]. Protollin™ [a non-covalent complex between PL from *Neisseria* species and *Shiguella flexneri* 2a LPS (1:1)] is an example that constitutes an attractive adjuvant for administration of different antigens. In animal studies and clinical trials Protollin™ have been shown to have strong adjuvant properties for bacterial and viral glycoprotein antigens [17,18]. A *V. cholerae* PL could be an alternative not only to protect against *V. cholerae*, but also as delivery system of non-related antigens and PAMPs as strategy to develop a combined formulation against enteric diseases preventable by the stimulation of the mucosal immune system, which constitute an advantage respect to others *V. cholerae* vaccine candidate including the licensed one. The use of PL by oral route is not recommended because they are less immunogenic certainly because gastric conditions of pH, temperature and proteases can affect proteins and liposomic structure [19], so nasal route has been used as an alternative with very good results [17,18,20].

In this paper we show that *V. cholerae* O1 derived components are assembled into a PL and intranasal immunization induces the same vibriocidal activity as 638 attenuated strain which has been used successfully in preclinical and clinical studies phase I [32].

2. Materials and methods

2.1. Bacterial strains and culture conditions

V. cholerae C7258, El Tor Ogawa [21] was used to obtain the PL. *V. cholerae* 638, El Tor Ogawa, attenuated strain vaccine candidate was used as a reference in the immunogenicity assay, while *V. cholerae* VC12, Classical Ogawa was used as an indicator strain in vibriocidal tests. The 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.

2.2. Preparation of outer membrane extracts (OME)

Cells were washed twice and adjusted to a final concentration of 210 mg biomass/mL in buffer solution 30 mM Tris–2 mM EDTA pH 8.5. The cells were then incubated in an ice water bath and SDS solution prepared to 2–15% was added to 0.1–0.5 mL/g biomass. After 1 h, the cells were centrifuged and the supernatant was enzymatically treated with DNAase and RNAase (Sigma, 5 μg/mL) for 1 h at 37 °C. Then, the preparation was centrifuged at 33,300 × *g* for 15 min at 4 °C and the supernatant was ultracentrifuged at 75,000 × *g* for 4 h, at 4 °C. The OME pellet was finally suspended in buffer solution 30 mM Tris–2 mM EDTA pH 8.5, filtered through a Sartorius Minisart-plus 0.45 and 0.2 μm filters and stored at 4 °C.

2.3. Photon correlation spectroscopy (PCS) and zeta potential analysis

This analysis were carried out using a Zetasizer 3000HS (Malvern Instruments, Malvern, UK). Vesicle size experiments were achieved using a solid-state laser as the light source. This laser is a nominal 4.5 mW laser diode with a maximum output of 5 mW at 670 nm. 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 ranged 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.

2.4. Chromatography

The OME (equivalent to 10 mg protein/mL, see Section 2.5) was applied onto a XK16/100 column (Pharmacia) packed with Sephachryl S-1000, previously equilibrated with buffer solution 30 mM Tris, 2 mM EDTA and 0.5% SDS pH 8.5. The chromatography separation was carried out at a flow rate of 0.4 mL/min (12 cm/h) using an Uvicord SII (280 nm, Pharmacia) and a Recorder Rec-102 (Pharmacia). For each chromatographic peak, the relationship between elution volume (ev) and total volume (tv) of the column was calculated. This relationship was used to estimate the molecular size of each peak.

2.5. Protein, LPS and membrane phospholipids determinations

The concentration of protein in the samples (OME and the chromatographically separated peaks) was calculated by the Lowry method using bovine serum albumin (BSA) as a standard [22]. The quantity of LPS was estimated by the use of a Western blot-densitometry method [23]. The concentration of membrane phospholipids was evaluated using the mineralization method of Fiske and Subarrow [24].

2.6. SDS-PAGE

Samples were incubated at 100 °C for 2 min in the presence of 2β-mercapto-ethanol (Merck, Germany) and separated by SDS-PAGE (acrylamide 12.5%) followed by a R250 Blue Coomassie stain [25]. Molecular weight markers consisted of a mixture of phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactoalbumin (14.4 kDa). The gel was placed on an image processor (Gene Genius Bioimaging System, Pharmacia) to determine the molecular size of the components and to identify the predominant proteins in each sample.

2.7. Western blot

An anti-*V. cholerae* O1 monoclonal antibody panel (produced at Finlay Institute, Havana, Cuba) was used, that included an

anti-toxin corregulated pili (TCP) (10E10E1) [26], anti-mannose-sensitive haemagglutinin (MSHA) (2F12F1) [27], anti-CTB (4E1G5), anti-CTA (1G10G5) [28], anti-Ogawa LPS (2B4G5) [29], and anti-OmpU (9H12E6) [30]. The procedure was followed as described by Burnett [31]. Preparations were processed by electrophoresis (SDS-PAGE, acrylamide 12.5%), transferred to a nitrocellulose and incubated with the monoclonal antibody panel as previously described. An anti-mouse peroxidase conjugated IgG (Sigma Chemical Co., St. Louis, MO) and diaminobenzidine as the substrate were used to develop the blot.

2.8. Scanning electron microscopy

OME was diluted in 0.1 M ammonium acetate pH 7.0 (Sigma) until 0.05 mg/mL and a drop placed onto cover slips, fixed with 5% (v/v) glutaraldehyde for 2 h, washed and then post-fixed with osmium tetroxide for 1 h. Then, the sample was dehydrated in an ascending ethanol series (70% 2 min, 96% 2 min, and absolute 10 min) and dried with hexamethyldisilatzane overnight. Microscopic examination was carried out with a ZEISS DSM-962 scanning electron microscope at 10 kV (Germany).

2.9. Immunogenicity assay

BALB/c mice (6–8 weeks old, females, CENPALAB, Cuba) were used in this study. OME and the chromatographically separated peaks were adjusted to 1 mg of LPS/mL. Each mouse received 25 μL (12.5 μL per nostril) by the intranasal route without anesthesia. Mice were immunized at days 0 and 28 (T0 and T28) and bled for serum collection at days 14 (T14), T28, T35, T42, T49, and T56 from T0 by retro-orbital puncture. In addition, one group of mice received PBS as placebo and another group received 25 μL 10^8 CFU of attenuated 638 strain vaccine candidate [32] as a reference. Bacteria were grown, cells harvested by centrifugation, washed and suspended to the above cell density in PBS pH 7.4. Sera samples for each group were collected, pooled and stored at −20 °C for subsequent analysis.

2.10. Determination of antibodies by ELISA

Anti-Ogawa LPS IgG antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) with *V. cholerae* C7258 Ogawa LPS as the solid-phase antigen. A positive titre was defined as the highest serum dilution, determined by interpolation, giving 0.4 units of absorbance above the background. The results were expressed as the logarithm of the reciprocal arithmetic media titre (geometric mean) for each serum [33]

2.11. Vibriocidal antibody assay

Serum vibriocidal antibodies were determined as described previously [33]. Briefly, 50 μL of two-fold dilutions of decomplemented 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% guinea pig complement (w/v) and placed in 96-well microtitre 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 color change of medium, which indicated bacterial dextrose consumption. During the bactericidal reaction the serum dilution on the first column of the plate was 1:20 and in the last one was 1:10,240. The vibriocidal antibody titre was calculated as the inverse of the highest dilution of serum causing complete inhibition of bacterial growth (no change of colour in medium). The results were expressed as $\log_{10}$ titre.

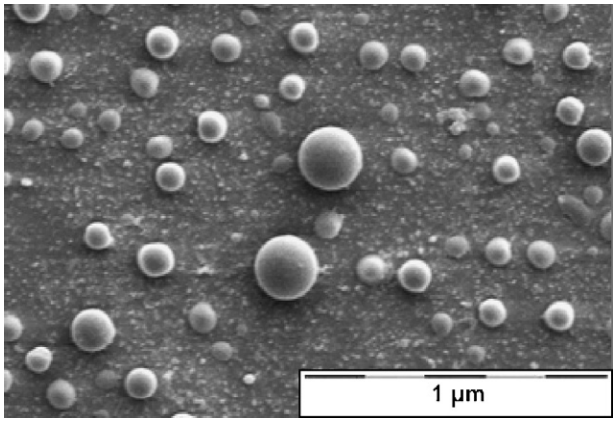

Fig. 1. Scanning electron microscopy of OME from *V. cholerae* O1, El Tor Ogawa, C7258 strain. Examination was done with a ZEISS DSM-962 at 10 kV. Vesicles of different sizes are observed using ×1000. The particle size (nm) was calculated in a range of 165.42–174.89.

2.12. Statistical methods

Parametric ANOVA (with repeated measurements) and *post hoc* associated multiple comparison procedures were used to analyze the data.

3. Results

Following a previously reported procedure [12] SDS was used to obtain PL from a virulent strain of C7258, *V. cholerae* O1, El Tor Ogawa. Physico-chemical characterization was done to elucidate the presence of PL in the OME prior to immunological studies.

3.1. Structural characterization of the OME

Scanning electron microscopy was carried out on the OME and we observed vesicles of different sizes (Fig. 1). The particle size of the vesicles was then determined using dynamic light scat-

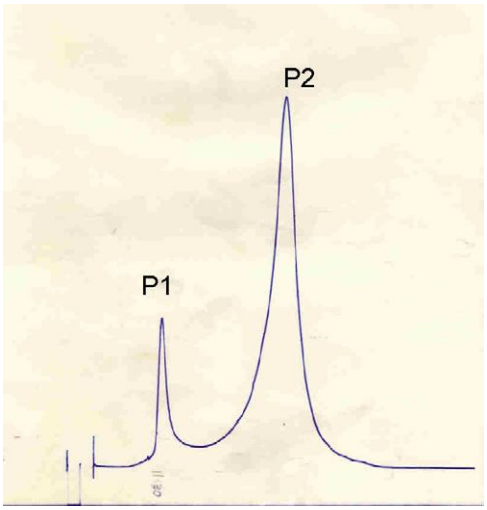

Fig. 2. Chromatography. The chromatography was carried out using a XK16/100 column packed with Sephachryl S-1000, previously equilibrated with 30 mM Tris–2 mM EDTA–0.5% SDS pH 8.5 buffer solution and with the following parameters—sample: outer membrane extract; concentration: 10 mg of proteins/mL; volume: 3.6 mL; flow rate: 0.4 mL/min (12 cm/h); instrumentation: Uvicord SII (280 nm) and Recorder Rec-102.

Table 1
PCS and zeta potential analysis.

Protein (mg/mL)	Volume (μL) in 5 mL of NaCl 1 mM	Size (nm)	Zeta (mV) ± S.D.
0.05	5	170.30 ± 4.59	−25.2 ± 0.71
0.15	5	169.27 ± 3.85	−23.8 ± 1.21

Size and zeta potential determined on samples suitably diluted with NaCl 1 mM at two different protein concentrations 0.05 and 0.15 mg/mL. Ten measurements were carried out for each experiment. Data are expressed as mean value ± standard deviation.

tering (Table 1) with a Zetasizer 3000HS (Malvern Instruments, Malvern, UK), which revealed a size average of 169.27 ± 3.85 nm. We also determined that the vesicle surface was negatively charged (−23.8 ± 1.21 mV, Table 1). The polydispersity index was 0.410, characteristic of a slightly heterogeneous system. To corroborate this we applied the OME onto a Sephacryl S-1000 chromatographic column and the absorbance recorded to 280 nm. Results (Fig. 2) showed two chromatographic fractions: named peak 1 (P1) and peak 2 (P2).

3.2. Composition of chromatographic fractions

Protein, LPS and membrane phospholipids determinations reveals that P1 contain more LPS (0.46 ± 0.06) than P2 (0.25 ± 0.07). Though, P1 and P2 did not showed significant different ($p < 0.05$) in regard to phospholipids content (0.25 ± 0.15 and 0.32 ± 0.11, respectively) (Table 2).

3.3. Identity analysis of main antigens on PL

Considering the microscopy and chromatographic results, we named the various components of OME in further as total PL (tPL). Fig. 3 shows the electrophoretic profile (PAGE, acrylamide 12.5% followed by a Coomassie R250 stain) of the tPL, P1 and P2. Several protein bands with molecular weights between 14 and 94 kDa were observed in tPL and P2. Nevertheless P1 seems to be almost totally (95–98%) composed of a single protein band of approximately 38 kDa, according to data from ImageMaster 1D elite V3.01 software Pharmacia. This protein is also represented in P2.

To determine if the 38 kDa protein corresponds with OmpU, a putative *V. cholerae* porine [34], a Western blot assay using an anti-OmpU monoclonal antibody was performed. Fig. 4A shows an intense reactivity of all the samples, particularly P1, with the anti-OmpU monoclonal antibody close to the 38 kDa band, according to the ImageMaster 1D elite V3.01 software Pharmacia reactive

Table 2
Protein, LPS and membrane phospholipids determination.

Samples	Protein (mg)	LPS (mg)	Phosp. (mg)
OME	1	0.28 ± 0.06[a]	0.62 ± 0.12[a]
P1	1	0.46 ± 0.1[b]	0.25 ± 0.15[b]
P2	1	0.25 ± 0.07[a]	0.32 ± 0.11[b]

Quantity of LPS was estimated from the OME and fractions reactive profile in western blot [using a monoclonal antibody anti-Ogawa LPS (2B4G5)] evaluated by densitometry with Ogawa LPS (1 mg/mL) quantified by dry weight as reference. The concentration of membrane phospholipids was evaluated using the mineralization method of Fiske–Subarrow. Different letters indicate significant differences using *post hoc* associated multiple comparison test ($p < 0.05$).

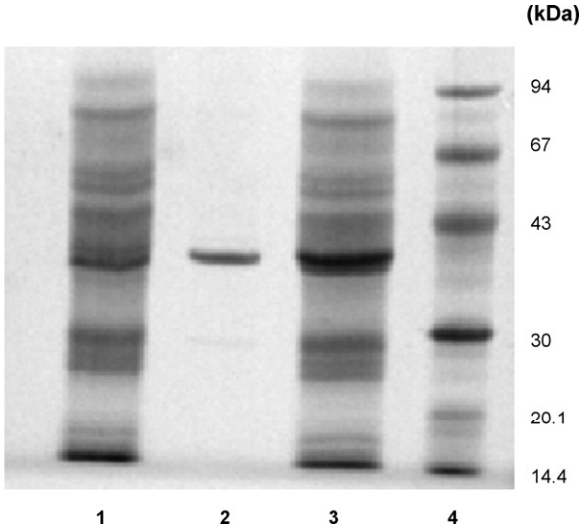

Fig. 3. SDS-PAGE (acrylamide 12.5%) followed by a R250 Blue Coomasie stain. The samples were applied using an amount of protein corresponding to: 10 μg of tPL, 4 μg of P1 and 7 μg of P2. The samples were incubated at 100 °C for 2 min in the presence of 2β-mercapto-ethanol (Merck). Lane 1: P2, Lane 2: P1, Lane 3: tPL, Lane 4: WMP [phosphorilase b (94 kDa), BSA (67 kDa), OVA (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa)].

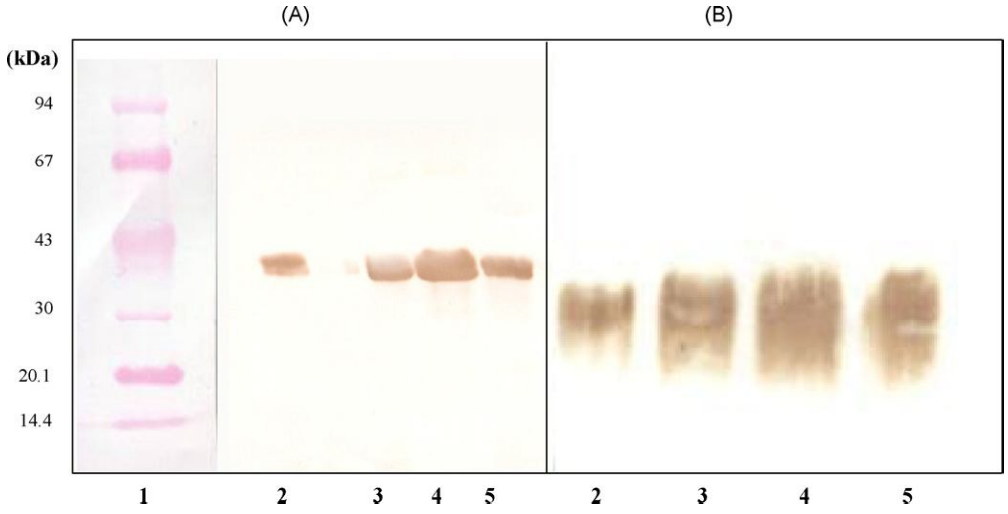

Fig. 4. Western blot. (A) Using anti-OmpU *V. cholerae* O1 monoclonal antibody; (B) using anti-Ogawa LPS *V. cholerae* O1 monoclonal antibody. Lane 1: MWP, Lane 2: El Tor Ogawa *V. cholerae* O1 C7258 wild type strain, Lane 3: tPL, Lane 4: P1, Lane 5: P2. On every Western blot samples were applied using an amount of protein corresponding to: 10 μg of tPL, 5 μg of P1 and 8 μg of P2.

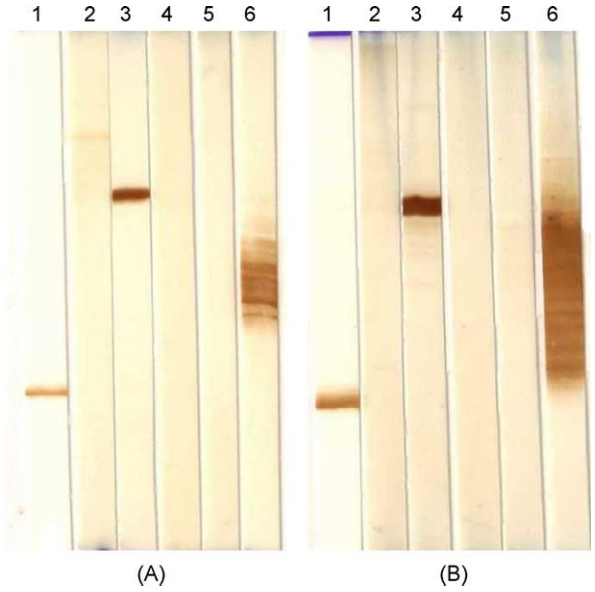

Fig. 5. Western blot of C7258 cell lysate (A) and tPL from the same strain (B) with different MAbs against relevant antigens of *V. cholerae* O1. Lane 1: MAb 2F12F1 (anti-MSHA); Lane 2: MAb 10E10E1 (anti-TCP); Lane 3: MAb 9H12E6 (anti-OmpU); Lane 4: 4E1G5 (anti-CTB); Lane 5: MAb 1G10G5 (anti-CTA); Lane 6: MAb 2B4G5 (anti-LPS Ogawa).

profile. Thus, we can conclude that OmpU is the major protein in all preparations. Fig. 4B also shows an intense reactivity of all the bands close to the 30 kDa band but with the anti-Ogawa LPS monoclonal antibody. Again this reactivity was more intense against the P1 preparation. Fig. 5 shows the Western blot assay of a C7258 strain cell lysate (A) and tPL from the same strain (B) with different monoclonal antibodies against relevant antigens of *V. cholerae* O1. Besides reactivity to anti-OmpU monoclonal antibody, anti-MSHA and anti-LPS monoclonal antibodies reactivity were observed in both preparations. No reactivity of anti-TCP, anti-CTB and anti-CTA monoclonal antibodies was seen.

3.4. Systemic immune response induced by intranasal immunization with tPL

BALB/c mice were immunized by the intranasal route with each preparation adjusted to 1 mg of LPS/mL. Another group of mice was immunized by the intranasal route too, but using 10^8 CFU of the attenuated *V. cholera* 638 strain as a positive control in this assay. The anti-Ogawa LPS IgG response was measured by ELISA using purified LPS as the coating antigen. A high antibody response was observed (Fig. 6) in all the immunized groups with no significant differences amongst them ($p < 0.05$). After the first dose (T0), there was no response until day 28 (T28), when a significant response ($p < 0.05$) was observed in mice immunized with the attenuated 638 strain (2.219). The peak response in all groups occurred 42 days (T42) after the first dose [3.36 (tPL), 3.25 (P1), 3.23 (P2) and 3.53 (638)], with no significant differences amongst them, although in T56 the response was still high in all groups [3.02 (tPL), 3.01 (P1), 2.84 (P2) and 3.15 (638)], with no significant difference compared with T42. No response was found in the placebo group at any time.

The vibriocidal antibody response in the serum of mice immunized with tPL, P1 and P2, and with the attenuated 638 strain (as reference) was also evaluated in this study. A high response was observed in all groups (Fig. 7) although the response of mice immunized with tPL (3.7), P2 (3.4), and 638 (3.5) was significantly ($p < 0.05$) higher (with no significant differences amongst them)

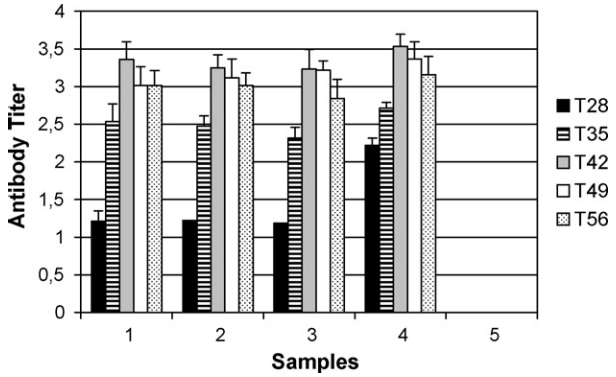

Fig. 6. Anti-Ogawa LPS IgG antibodies response in adult mice after two intranasal immunizations (0 and 28 days or T0 and T28). Sample 1: tPL, Sample 2: P1, Sample 3: P2, Sample 4: 638 attenuated strain, Sample 5: placebo. The results are expressed as the logarithm of the reciprocal arithmetic media titer (geometric mean) for each serum. No response was detected for any sample at T7, T14 or T21.

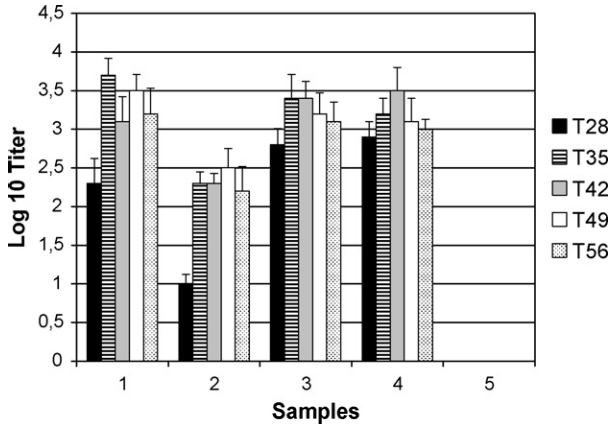

Fig. 7. Vibriocidal antibody titer in the serum of adult mice after two intranasal immunizations (T0 and T28). Sample 1: tPL, Sample 2: P1, Sample 3: P2, Sample 4: 638 attenuated strain, Sample 5: placebo. The titer was calculated as the inverse of the highest dilution of serum causing complete inhibition of bacterial growth. The results were expressed as $\log_{10}$ titer. No response was detected for any sample at T7, T14 or T21.

when compared with the response to P1 (2.5). In a perfect correspondence with the results of the ELISA, mice immunized with attenuated 638 strain or P2 showed a peak of vibriocidal response in T42, while mice immunized with tPL and P1 had their peaks in T35 and T49, respectively with no significant differences with other times. No activity was detected in the pre-immune serum (pre-immune titer <25) or placebo group.

4. Discussion

In this paper, we have characterized PL obtained by detergent extraction from the outer surface of C7258, *V. cholerae* O1, El Tor Ogawa strain as part of strategy to develop a vaccine formulation against enteric diseases preventable by the stimulation of the mucosal immune system.

Scanning microscopy revealed that the OME consists of PL of different sizes which correspond with results of molecular exclusion chromatography where two different sized peaks were obtained. Spectroscopic analysis of OME or tPL showed a vesicle average size of 169.27 ± 3.85 nm and a slightly high polydispersity index (0.410), confirming differences in the size of the chromatographic peaks P1 and P2. It has been described that particles about 5 μm are retained within the Peyer's patch and anything smaller than this will travel

to the lymphatics [35]. This suggests that the size of the antigen can determine the nature of an immune response and that larger antigens held within the Peyer's patch stimulate local mucosal immune responses while smaller antigens escaping to the peripheral lymphatic will induce systemic immune responses [36].

Evaluation of zeta potential of tPL show that vesicle surfaces are negatively charged (-23.8 ± 1.21 mV), which is important for the stability of PL because electrostatic repulsion forces can prevent undesirable aggregation and/or precipitation of PL.

P1, P2 were further analyzed to elucidate the protein, LPS and phospholipids composition. The results showed that the distribution of these components for each sample was different. P1 had more LPS (0.46 mg) than P2 (0.25 mg), though both have similar phospholipids (0.25 mg and 0.32 mg, respectively). Further studies using SDS-PAGE and Western blot showed that the proteins were not equally distributed in P1 and P2. Interestingly, P1 had only one protein with an estimated molecular mass around 38 kDa, which was reactive against monoclonal antibody anti-OmpU in a Western blot. It is important to note that these results have been found in several lots of *V. cholerae* OME showing the consistency of detergent extraction process and differences on peaks depends mainly on the kind of detergent used during the process (SDS) more than the process itself [12]. So the segregation of protein, LPS and phospholipids in two different peaks is an interesting phenomena but their explanation is beyond the scope of this work.

OmpU has been reported as one of the major *V. cholerae* outer membrane proteins [37] and its molecular mass has been estimated to be 38 kDa by SDS-PAGE when the sample was incubated at 100 °C for 2 min [38]. Sperandio et al. in 1995, reported OmpU as a potential adherence factor when *V. cholerae* adheres to HeLa, HEp-2, Caco-2, and Henle 407 cells, which was inhibited by anti-OmpU serum or $F(ab')_2$ fractions [34]. Cloning and molecular characterization of the OmpU genes have been carried out at the same time as functional characterization of the protein [39,40]. These studies have revealed some homologies of OmpU with porins from *E. coli* and other species [39,41]. Further studies will be design to evaluate cross reactivity of antigens on tPL from *V. cholerae* with antigens from other enteropathogenic bacteria.

We also found LPS and MSHA as the other PL relevant antigens. LPS induces protective immune responses in humans and animals [42–44] and thus its use as a protective immunogen for *V. cholerae* vaccine development has been widely accepted [44–46]. Anti-LPS antibodies are mainly responsible for vibriocidal activity of sera. Vibriocidal assay records killing of *V. cholerae* cells in presence of immune sera and complement [47]. Mosley et al. [48] provided evidence for the involvement of systemic antibodies in protection against *V. cholerae* and demonstrated a correlation between serum vibriocidal antibodies level and protection.

MSHA is a controversial antigen with respect to its role in a protective immune response. Nevertheless, it has been established that polyclonal anti-sera and one anti-MSHA monoclonal antibody protected infant mice and prevented fluid accumulation in rabbit ileal loops [49,50]. A MSHA defective mutant showed reduced colonization in adult rabbits suggesting that this pilus may play an important role in intestinal colonization [51].

The best characterized anti-bacterial immune response induced by *V. cholerae* infection is the serum vibriocidal antibodies. This response is primarily directed against *V. cholerae* LPS, but may also include activity against outer membrane proteins [52].

Nasal immunization was carried out using two doses 28 days apart of samples (tPL, P1, P2) with 25 μg of LPS each one and the 638 strain attenuated vaccine candidate. The fact that vibriocidal responses in mice immunized with P1 is lower than that observed in other groups (tPL and P2), could be contrasted with results shown in Fig. 6 (anti-LPS Ogawa IgG). Mainly because it is known that anti-LPS antibodies are responsible for vibriocidal responses [43]. Nevertheless, P1 is basically composed of LPS and OmpU, while in tPL, PL2, and the 638 strain the antigenic repertoire is wider which favors the response capacity, not only because of the bigger availability of antigens that could cause vibriocidal responses but because of the synergism among them and the consequent response enhancement. An interesting feature of PL is their multiantigenicity, protection could be obtained by a cooperative effect of the responses to multiple antigens and PAMPs, each of which may be insufficient to confer immunity when administered alone.

Vibriociodal responses observed in mice immunized with attenuated 638 strain by the intranasal route in this study, is proportional with the vibriocidal response observed by Silva [53]. These authors administered, intranasal, four doses of 638 attenuated strain (T0, T28, T42 and T56) to adult Swiss mice and monitored the vibriocidal immune responses in T25, T35, T49 and T63. The response observed by them in is comparable with that observed by us. Thus we have demonstrated that immunization with tPL from *V. cholerae* by the nasal route induces the same vibriocidal activity as the positive control group 638 attenuated strain which has been used successfully in preclinical and clinical studies phase [32,33].

Indeed different *V. cholerae* vaccines are administered orally, based on the demonstration that a correct stimulation of the mucosal immune system was the best way to achieve an adequate protective immune response against a pathogen like *V. cholerae*. However, we are trying to develop a nasal vaccine. Maybe this could be wrongly interpreted, but it would be important to take into account the reasons we considered: (i) PL has no shown an elevated response when administered orally to mice (with the exception of higher doses), we thought it may be due to the fact that PL is constituted of OMPs extracted from *V. cholerae*, which are very vulnerable to high levels of intestinal proteases that would significantly affect PL composition and therefore its protective immune response. We know that Protollin™ is immmunogenic when administered either intranasal or intragastric in mice [54], but the intragastric route demanded more amount of antigen. On the other hand, (ii) intranasal route has been successfully used lately to generate a potent immune response at mucosa level, in addition several authors have stated that it is more effective than the oral one since it not only generates a higher response at mucosa level but also it does at peripheral level. Besides, we consider that intranasal administration of *V. cholerae* PL on clinical study is possible taking into account that Protollin™ demonstrated to be safe in doses up to 1.5 mg (contained approximately 700 μg of LPS of *S. flexneri* 2a [15,55]), nevertheless toxicity studies in preclinical stage must be done.

Finally the adjuvant potential of our candidate is ready to be tested with antigens from enteric pathogens non-related with *V. cholerae* O1, as well as its transformation into a cochleate structure, which could increase the quality of the formulations in terms of stability and immunogenicity [56].

5. Conclusion

Two populations of PL were obtained P1 and P2, which showed a high capacity individually and together (tPL) to generate a significant immune responses against *V. cholerae* O1 in mice using the intranasal route. In addition, we demonstrated the importance of LPS and total proteins in the tPL extract to induce vibriocidal activity. If we consider the vibriocidal antibody level to be the best marker of *V. cholerae* immune responses that correlates with protection in humans, then the comparable results obtained in BALB/c mice immunized with PL and the 638 attenuated candidate vaccine shows huge potential. Although a lot of work has been reported

on liposome characterization and *V. cholerae* immunogenicity, few have proposed strategies to characterize detergent extracted PL from a natural source and none the benefits of using one derived from *V. cholerae* to induce vibriocidal activity similar to an attenuated vaccine candidate. We therefore believe that immunogenic *V. cholerae* PL could be a key issue to develop a combined formulation against enteric diseases.

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