



## Remote induction of cellular immune response in mice by anti-meningococcal nanocochleates - nanoproteoliposomes

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**1 Remote Induction of Cellular Immune Response in Mice by Anti-meningococcal**  
**2 Nanocochleates – Nanoproteoliposomes**

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## Abstract

New adjuvant formulations, based on proteoliposomes <40 nm and cochleates <100 nm, without Al(OH)<sub>3</sub> adjuvant, were evaluated regarding their ability to generate Th1 immune response through a Delayed -Type Hypersensitivity Test, at the mouse model, by using a *Neisseria meningitidis* B protein complex as antigen. The formulations were administered by intramuscular (IM) (2 inoculations - at baseline and after 14 days) and intranasal (IN) (3 inoculations at 7 days) immunization pathways. All IM immunized groups were able to induce similar response to these formulations as well as to VA-MENGOC-BC® vaccine - containing Al(OH)<sub>3</sub> adjuvant (used as positive control of the trial). In all groups, the induced inflammation (IP) rate was statistically higher than in the negative control group (CN) (p<0.05). Immunogenicity, measured by HSR and CD4<sup>+</sup> lymphocyte increase was equivalent to the control vaccine and most important, granuloma reactogenicity at the site of injection was eliminated, fact demonstrated by histological study. All groups of animals immunized by IN route showed HSR reactions and statistically significant differences with respect to the CN group. However, IP values were lower, with statistical differences (p<0.05) for the same adjuvant formulation IM administered, except the AIF2-nCh formulation that generated statistically similar induction (p>0.05) by both immunization pathways, suggesting it to be the best candidate for the next IN trial. Proteoliposome and cochleate formulations tested were able to mount potent Th-1 immune response, equivalent to the original vaccine formulation, with the advantage of less reactogenicity in the site of the injection, caused by the toxicity of Al(OH)<sub>3</sub> adjuvant gel.

**Key words:** Delayed-Type Hypersensitivity; Nanoproteoliposomes; Nanocochleates; adjuvant; anti-meningococcal vaccine B; TCD4<sup>+</sup>.

## 1. Introduction

The rational design of vaccines constitutes one of the main strategies to fight against complex diseases that beset humanity. However, the scarcity of adjuvants with immune-potentiating capacity, which at the same time provide a suitable release of antigen *in vivo*, has considerably limited the development of new effective vaccines (Singh et al., 2007).

The Cuban anti-meningococcal vaccine VA-MENGOC-BC® has been one of the most efficient approaches to protect against B and C meningococci, with over 60 million doses administered in humans (Vermikos and Medini, 2014). This vaccine is formed by proteoliposomes (PL) derived from the *Neisseria meningitidis* serogroup B (NMB) external membrane, which are the main antigenic component, and the purified capsular polysaccharide of serogroup C, whose components are absorbed in aluminum hydroxide gel (Sierra et al., 1991; 2000). The latter has been a broadly used and approved adjuvant for more than one century in human vaccines. However, its immuno-potentiating capacity directs the immune response toward a predominating type-Th2 pattern, and it is useful only in parenteral applications, fundamentally by the intramuscular route (Pérez et al., 2007). It has demonstrated in general a good safety record and strong antibody induction, but because it is not suitable for inducing optimal type-Th1 or cytotoxic (CTL) responses, its use is appropriate when the antibody systemic response is sufficient to provide immunity (O'Hagan and Valiante, 2003) but inappropriate when a potent type-Th1 or CTL response is required to provide immune protection. Another of its disadvantages is reactogenicity due to the formation of granulomas resulting from the persistence of alumina in the injection site (Kurella et al., 2000). Studies focusing on vaccines show the paramount importance of the connection between immunogenicity and reactogenicity (Bracho et al., 2006; Girard et al., 2007; Lastre et al., 2006). For this reason, particulate vaccine formulations have been designed which do not require the additional use of this type of aluminum-derived adjuvant.

At Finlay Institute, Cuba, structures have been obtained with PL and adjuvant properties starting from NMB external membranes, as well as cochleates (Ch) derived from the interaction of the anionic lipid vesicles of the PL with divalent cations such as calcium, *inter alia* (Pérez et al., 2008). This type of structure has demonstrated immune-potentiating and immune-modulating properties, as well as capacity to act as slow-release systems that transform them into good adjuvant candidates (Pérez et al., 2007). Previous studies have demonstrated the capacity of the PL to induce a pattern of cellular immune response (Pérez et al., 2006), as well as their potential to be used as adjuvant in anti-allergy vaccines (Lastre et al., 2006).

Furthermore, a PL derivatives was obtained, which was then used in anti tumoral immune therapies, increasing the immunogenicity of highly tolerated gangliosides (Mesa et al., 2006). Its strong capacity to activate dendritic cells and macrophages has been demonstrated (Rodríguez et al., 2005). The Ch have also been demonstrated to have immune-stimulant properties comparable with PL, although the latter manifests inherent properties of this structure type regarding antigen release (Zayas et al., 2006). Results reveal that the Ch also stimulate a pattern of cell-type immune response, thus allowing them to be used as immune-modulating and release systems for the development of mucosa vaccines, particularly nasal ones (Del Campo et al., 2010); a promising application for this was tested in an anti-malaria vaccine (Bracho et al., 2009). The incorporation of new antigens into this structure suggests its use as an immunization adjuvant, with a wide variety of pathogen derivative antigens and through different administration paths (Bracho et al., 2006). In particular, the Ch have been shown to be more potent as a mucosal adjuvant, given the increase of their stability in the mucosa (Pérez et al., 2006).

During the past decade there have been many examples of pre-clinic studies that reveal promising results of this PL, and of the Ch derived from them, indicating that they can work

as an adjuvant both in parenteral and mucosal vaccines when inducing a type-Th1 immune response (Acevedo et al., 2009; Del Campo et al., 2006; Rodríguez et al., 2005), as well as both systemic and mucosal antibody responses (Campo et al., 2009). However, the major drawback of these adjuvants is that the antigen must be incorporated during the manufacturing process (Pérez et al., 2007). This implies certain limitations including the decrease in homogeneity during antigen distribution in Ch and the poor efficiency of the process in which the mentioned structures are identified (Pérez et al., 2004). These inconveniences are minimized when PL and Ch are formed starting from purified phospholipids in specific quantities, to which are added specific molecules considered necessary for designing a formulation. On the other hand, when building *ad libitum* nanoparticles, more convenient sizes can be designed based on their immune-pharmaceutical properties, such as those that spontaneously result from re-conformation, of reordered protein membranes.

Considering the importance of inducing protective immune responses, stress has been placed on its polarization toward a Th1 lymphokine pattern (Pérez et al., 2008). This explains the importance of evaluating the cellular immune response in the study of the new vaccine candidates, especially if they concern the eradication of intracellular pathogens and of tumor cells (Abdel-Daim et al., 2018). The functionality of activated Th1 clones as a result of the immunization and the immunity mediated by cells can be tested *in vivo*, using the pattern of delayed-type hypersensitivity response (HSR). This latter constitutes an expression of the activation of specific and unspecific protection mechanisms and of immunologic memory induction, a process similar to the cell-mediated immunity characterized by the expansion of specific T cells (Kobayashi et al, 2001).

The activation of T cells for the antigen cellular presentation releases cytokines and chemokines, which stimulate the vascular endothelial cells to increase permeability and

induce phagocyte infiltration and the accumulation of body fluid in the HSR reaction site (Kraneveld et al, 1995). The HSR assay is an unequivocal *in vivo* indicator of Th1 response driven by cell-mediated immunogenicity. HSR is usually applied to determine latent infection by tuberculosis (Walsh et al., 1995), and it is characterized by induration in the antigen intradermic injection site with a lapse of 48 to 72 hours after its administration, reflecting the infiltration of memory mononuclear cells that are antigen specific (Stewart et al., 2006).

This study evaluated new adjuvant formulations, based on PL and Ch, of smaller sizes than previously reported, and without aluminum hydroxide. An antigen (NMB external membrane proteins, extracted without forming vesicular structures) was used for this purpose. Through the HSR response pattern study, we showed the ability to stimulate an immune cellular response both via intramuscular and intranasal inoculation routes in BALB / c mice.

## **2. Materials and methods**

### **2.1. Adjuvant formulations**

The adjuvant formulations were designed using, as an antigen, a complex of NMB proteins extracted from bacteria (the extraction was carried out using a mild DOC detergent method and the detergent was removed with ethanol; the latter was in turn removed using the filtration method) (Bracho et al, 2006) and purified without forming vesicular structures, which in this article are identified as differentiated proteins (PD). They are combined with immune-modulating components of a defined molecular structure, to produce two formulation groups (1 and 2) that are structured in neo-proteoliposome (nPL) and neo-cochleate (nCh) forms. The obtained formulations were named AIF1-nPL, AIF1-nCh, AIF2-nPL and AIF2-nCh, (AIF: Adjuvant IFAL – “Finlay”). Liposomes were developed starting from a 1,2-dioleoylphosphatidylserine (DOPS) mixture and cholesterol (Sigma Aldrich Co.), using the procedure of dehydration-rehydration (Kirby and Gregoriadis, 1984). The Ch were obtained

by slow addition of calcium chloride (0.1 M) to the suspension of pre-formed liposomes (Zarif and Mannino, 2000). Both liposome and cochleate preparations were stored at 4°C and protected from light. A suspension buffer of the PD protein complex (Tris 2mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® and detergent pH 7.4) was used as control-denominated PD-Tp, a preparation of NMB vesicles of external membrane (VME), in a similar buffer solution using as detergent Sodium Deoxicholate (DOC) and the Cuban anti-meningococcal vaccine, VA-MENGOC-BC® from Finlay Institute.

The 4 formulations to be tested and the 3 control formulations are described in detail as follows:

1. Type: PL as immunomodulator: AIF1nPL- 15µg protein PD (VME) in 25 µL buffer Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as detergent, pH 7.4 + integral LPS 1%.
2. Type: cochleate as immunomodulator: AIF1nCh-5µg protein PD (VME) in 25 µL buffer Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as detergent, pH 7.4 + integral LPS 1%.
3. Type PL as immunomodulator: AIF1nPL- 15µg protein PD (VME) in 25 µL buffer Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as detergent, pH 7.4, without integral LPS.
4. Type cochleate as immunomodulator: AIF2nCh-15µg protein PD (VME) in 25 µL buffer Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as detergent, pH 7.4, without integral LPS.
5. Type Control (soluble): PD-Tp-15 µg protein PD (VME) in 2 5µL buffer Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as detergent, pH 7.4, without integral LPS and without forming cochleate or PL.



6. Type Control for intra muscular applications: VBC-Va-Mengoc-BC (Bracho et al, 2006).

7. Type Control for intra nasal applications: VME-Va-Mengoc-BC, as recommended by Bracho et al, 2006, but without  $\text{Al}(\text{OH})_3$  gel.

## **2.2. Characterization of the nanoproteoliposomes and nanocochleates using electronic microscopy**

### *Electronic Transmission Microscopy*

The electronic images were obtained by Electronic Transmission Microscopy with a CM12 Phillips microscope. The samples were processed by negative staining using the traditional drop method. After the adsorption of 5  $\mu\text{L}$  of the nPL and nCh formulations on a grill covered with coal, the samples were tinted with phosphotungstic acid at 2% and observed at 100,000X magnification.

### *Scanning electron microscopy*

A drop of each different formulation was applied on a glass microscope slide and put to dry at room temperature for a period of 12 hours. Later on, they were covered with a fine layer of gold-palladium and loaded in a LEICA 440 scanning electron microscope. The images were observed with magnification ranging from 15X to 290,000X and a 4.5 nm resolution.

## **2.3. Animals used in the experiment**

The number of animals (BALB/c mice) required was calculated before the study, taking into account all the tests we were considering at that time; the mice were brought from the Center for the Production of Laboratory Animals, Cuba (CENPALAB, breeding-production facility) and had approximately identical characteristics in terms of isogenic origin, sex (males), age (6-8 week old) and, implicitly, weight, etc. To assure complete homogeneity, random lists

were generated, with the formulations assigned per animal according to the order of extraction of each animal from the cages. Care to mice was assured, according to established institutional norms set in the Guide for the Care and Use of Laboratory Animals, 8<sup>th</sup> Edition, 2011, from the Institute for Laboratory Animal Research, Division on Earth and Life Studies, National Research Council, of the National Academies (<http://www.nap.edu>) under free-of-pathogen micro isolation conditions. These experiments were carried out with authorization and approval from the Ethics Committee of Laboratory Animals of CENPALAB. The design, implementation and evaluation of research that involved using experimentation animals abided by the general bioethics principles of refinement, replacement and reduction (RRR), as well as by laboratory and laboratory-animal-handling good practices (Hernández, 1997).

#### **2.4. Study description**

A total of 65 animals were used, which were randomly distributed into 13 groups (5 animals per group). Formulations were administered both by the intramuscular (IM) as well as intranasal (IN) immunization routes. The IM immunization schedule consisted of two inoculations, the first one at the beginning of the study and the second after 14 days. A 15 µg dose corresponding to the protein concentration was administered in 250 µL of saline phosphate buffer (PBS). Mice were inoculated using a deep intramuscular puncture in the left quadriceps of the hind limb. The IN immunization consisted of three inoculations, with intervals of 7 days. The animals received a 15 µg dose corresponding to the protein concentration in 25 µL of PBS (12.5 µL in each nasal fossa). Each experimental group was immunized with a trial formulation. The VA-MENGOC-BC® vaccine was IM administered (25 µg/ dose), while the VME of NMB was IN administered (15 µg/ dose), both as controls. A negative control group was also included to which PBS was administered; it is identified as CN.

## 2.5. Delayed-type hypersensitivity trial

After 21 days, all the animals received, by the intradermal immunization route, 15 µg of PD in 30 µL of PBS (0.5 mg/mL in PBS) in the left hind footpad, and 30 µL of PBS in the right one, the latter used as control. The absence of inflammatory reaction at 4 and 24 hours was confirmed. The width of the paws was measured in millimeters (mm) using a digital Vernier caliper, 0-150 mm (OMNI - TECH), between the third and fourth pulp-protuberances of both hind limbs, in order to measure the inflammation produced at 48 hours. The percent of induced inflammation (%IP), as a criterion of HSR reaction, was calculated according to the bellow formula (Lincopan et al., 2009), with results expressed as % IP ± the standard error of the mean (SEM).

$$\% \text{ IP} = 100[(\text{left hind footpad thickness}) - (\text{right hind footpad thickness})] / (\text{mean thickness uninjected left hind footpad}).$$

## 2.6. Histological study of hind extremities

The animals were dispatched by cervical dislocation at two days after the HSR trial. Macroscopic analysis of the right and left hind extremities was performed, and these were collected for the histological study. Tissues were set in 10% neuter formaldehyde for 24 hours and later on decalcified using the Lilies decalcifier. For tissue cuttings, a horizontal microtome (Histoside200) was used with a thickness of 4-6 µm. The samples, once extended in laminate, were tinted using hematoxylin-eosin and were observed in a simple microscope (Carl Zeiss). For each animal on which a necropsy was performed, 5 cuts were observed.

## 2.7. TCD4<sup>+</sup> Lymphocytes analysis by flow cytometry

The analyses were carried out using a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The TCD4<sup>+</sup> lymphocyte subpopulations were analyzed using the anti CD4<sup>+</sup> mouse rat monoclonal antibody, marked with PE-Cy5 (BD Bioscience Pharmingen). The samples were characterized according to the intensity of fluorescence radiated by the fluorochrome used in the markings as well as by rugosity and size of the cells. The obtained data were analyzed using the Cell Quest Program for McIntosh.

## 2.8. Statistical analysis

The statistical processing of the results was performed using the Statistica program, version 6.0, on Windows. The Kolmogorov-Smirnov test was used for assessing the normality of the distribution and the Levene test used to evaluate the homogeneity variance. A one-way variance analysis was used (ANOVA) by means of a Dunnet and Duncan test. In all the cases a  $p \leq 0.05$  significance level was used.

## 3. Results

The images of the PL and Ch used in formulations, obtained by both electronic transmission and scanning electron microscopy, are presented in **Figure 1**. It is possible to see the spherical shape of the PL (in images A and B of Figure 1) which is characteristic of this type of structure, as well as its homogeneous size. In addition, certain determination values of particle sizes are shown, which ranged from 10 to 40 nm. In the Ch that were formed by transformation of previously obtained liposomes through the addition of calcium salts, a cylindrical particle (figure 1C) can be observed. It is elongated and continuous, similar to a cigarette, with multiple layers. An example of the dimensions of the diameter of the cochlear structures obtained is shown: it is inferior to 100 nm on average.

With the aim of verifying the induction of cellular immune response when stimulated by immunization with the new anti-meningococcal adjuvant formulation variants via systemic

and mucosal pathways, an HSR trial was carried out. The non-presence of immediate hypersensitivity reactions was examined at 4 and 24 hours, finding induction of inflammatory processes after 48 hours, time at which the inflammation was measured (induration), caused in the hind limbs. A photo of the hind limbs of one of the animals vaccinated with the adjuvant formulations under study is presented in **Figure 2**. Increase in volume and reddening of the footpad in the left hind limb, which had been inoculated with the antigen, can be observed. In the right footpad, injected with a phosphate saline pH=7.4 (PBS) buffer, these inflammation symptoms did not appear.

The volume of the induration was determined measuring the width of the paws, using a digital Vernier caliper; the values obtained from these measures were correlated with the volume data (mL) of the inflammation taken from the limbs, using an automatic Plethysmometer, (Ugo Basile, Italy), (data on comparison not shown).

Both formulations based on nPL and nCh administered through each of the immunization routes generated HSR responses (**Figure 3**). All groups IM immunized (**Figure 3A**) were able to induce a similar response among them and with respect to the VA-MENGOC-BC® vaccine used as the trial positive control. In all groups, the %IP was statistically higher than the CN group ( $p<0.05$ ). In the group inoculated with PD-Tp, the %IP values were higher ( $p<0.05$ ), not only with regard to CN, but also compared to the rest of the groups. Likewise, all groups of IN immunized animals (Figure 3B), showed HSR reactions and statistically significant differences with regard to the CN group. However, the %IP values were smaller, with statistical differences ( $p<0.05$ ) for the same adjuvant formulation, intranasal administered with regard to the intramuscular one, except AIF2-nCh formulation that induces a similar induration from the statistical point of view ( $p>0.05$ ), through both immunization routes.

The afore mentioned results were verified with the histological studies of the anatomical regions closer to the inoculation site of the antigen stimulus. A sample coming from the hind limb against lateral where PBS was administered was used for control purposes. It was possible to confirm an abundant generation of inflammatory cells infiltrated at 48 hours of said stimulus which consisted, fundamentally, of abundant lymphocytes, other mononuclear cells such as macrophages, and some polymorphonuclear neutrophils and eosinophils.

When comparing the hind limbs in each animal, an inflammatory reaction was observed only in the limb inoculated with the antigen, and not in the one inoculated with the vehicle substance. In all groups the observed inflammation was related to an increase in the measured volume that, in turn, served as base for the determination of the %IP.

In each of the groups studied by both immunization routes, the intensity of the hypersensitivity reaction is similar to the %IP-measured values of the limb, and it also was equal to the degree of inflammation observed in the histological exam at the footpad level. The latter was classified according to the severity of the damage found in the tissue, with signs allocated (+ for slight level, ++ for moderate, +++ for strong, and ++++ for severe) that took into consideration the presence of edema, infiltration of inflammatory cells in the subcutaneous tissue, the appearance of thickening resulting from the activation of vascular endothelium, fibrin deposits, necrosis focus, *inter alia*.

The determination of delayed type hypersensitivity, respectively, the classification of DTH-R inflammation (according to the observation of different experimental groups), based on the degree of inflammation and infiltration (observed according to histopathology studies of foot lesions) is depicted in **Table I**.

The results of the HSR study, despite the fact that it does not show a statistical correlation, present a general tendency to correspond with the increase in the TCD4<sup>+</sup> lymphocyte clones. This is observable when using a flow cytometer to analyze these cell subpopulations in the

sub-lingual and cervical lymph nodes of animals immunized via the IM and IN pathways. If analysis is limited to cochlear formulations, the correspondence is stronger: the formula AIF2-nCh through IN shows the highest values of HSR and the highest values of TCD4<sup>+</sup> clones. The relative percentage of TCD4<sup>+</sup> lymphocytes was calculated in relation to total cells. We indicate that HSR results are statistically superior to negative control,  $p < 0.05$  and TCD4<sup>+</sup> are in the normal classification of this type of trial.

#### 4. Discussion

Leaving aside the mathematical correlation, we can say that there is an interesting correspondence between the results of the HSR, the inflammation due to the Th-1 immune response pattern and the increase of the TCD4<sup>+</sup> lymphocyte clones that are essential elements in its establishment and that correspondence is more easily distinguishable in the route of nasal immunization and with the formulation of Ch. That was also corroborated with the histopathologic studies.

The HSR is an inflammatory reaction mediated by cells, among them effector and memory T lymphocytes infiltrated in the inoculation site of an antigen, against which the immune system has already been previously sensitized. It can thus be a very valuable marker for functionality, as well as for cell-mediated immunogenicity. The inflammatory reaction is characterized by the reddening and swelling in the site of the antigenic challenge (Kraneveld et al., 1995).

The Th1 lymphocyte clones have the capacity of generating inflammatory processes which mediate this type of reactions when faced with a new antigenic challenge. The HSR is an immunologic response mediated by memory T lymphocytes that implies the recruitment of macrophages and other cells that, once activated, secrete pro-inflammatory cytokines. The activated cellular immune response increases the quantity of produced cytokines and chemokines, which favor monocyte, eosinophil and neutrophil recruitment at the site where

the antigen has been inoculated, against which the immune system has already been challenged (Posadas et al., 2007).

It is a beneficial response from the host that constitutes an adaptive protection mechanism or its maturation as immunologic memory. However, it can also cause tissue damage due to granuloma formation, depending on the intensity of the reaction (Kobayashi et al., 2001). The HSR response is a very convenient model to evaluate the effectiveness of *in vivo* cellular immune response induction.

In this study, the fact that the inflammatory reaction was visible as of 48 hours is very significant, since it is indicative of a late response, resulting from the recruitment of previously activated cells T at the time of the first immunization (vaccination) toward the challenge site, and therefore it confirms the development of the immunologic memory and the T cell response.

The %IP analysis demonstrated that, in each of the immunization routes, the formulations were always able to generate this type of response. This reaffirms results from previous studies, with either vesicular or proteoliposomic structures (Lastre et al., 2006). The histological confirmation resulted in a mobilization of immune-competent cells, and its intensity or severity was related to the activation level of specific lymphocytes for the said antigen (PD proteins). The inflammation degree reached in all cases corresponded to the prospective immunologic response pattern, in accordance with the design of each of the formulations.

When analyzing the behavior of the formulations for the intramuscular pathway, it can be observed that they were able to induce, in animals under treatment, a similar response to VA-MENGOC-BC®, even at a lower dosage than that used in this experiment for the VA-MENGOC-BC® immunization group. This vaccine has been used in millions of children in



several countries, with a high safety profile, demonstrating its protection capacity (Sierra et al., 1991; 2000). The results of the present research might be very useful, as they indicate possibilities for clinical applications of the new adjuvant formulations in the treatment of many diseases – not only meningococcal meningitis, but also cancer and infections caused by intra-cellular pathogens. Indeed, these formulations could, in the future, improve anti-meningococcal vaccine and also be used in vaccines that require induction of this type of immune-response pattern, avoiding at the same time the use of aluminum adjuvant.

The PD-Tp group immunized through the IM route showed significant differences with the rest of the groups, which was to be expected, since the formulation pertaining to this group has a 100% antigenic identity with the immunogen that was used in this trial. There are no statistically significant ( $p>0.05$ ) differences between the response of HSR induced by the formulations with proteoliposomal structure and that induced by the same formulation wherein liposomes have adopted a cochlear structure. This suggests that by the IM immunization route both structures induce a cellular immune response.

In the case of the IN immunized animals, it can be observed that all the groups showed a similar HSR reaction. The lower values obtained by this immunization group compared with the IM group are a logical result, obtained basically due to the anatomical distance and the immunological compartment that separates the site where the second antigenic challenge took place with regard to the immunization site.

Nevertheless, the IN group immunized with AIF2-nCh shows the highest inflammation percentage and differs from the rest of the groups. This formulation is similar to the particles in cochleate form, which suggests its use as a possible adjuvant candidate highly promising in mucosa vaccines. Other researchers have obtained similar results using different Ch as an adjuvant (Del Campo et al., 2006; 2010), including *inter alia*, Ch elaborated on a VME of

NMB basis, IN and intragastric administered, induce HSR reactions. Our results therefore coincide with that reported by Lycke (2004), when it outlines that both Ch and PL applied by the IN route are an effective strategy to stimulate immunity, in both a systemic and local way.

In this trial, the analysis of the relative percentage of TCD4<sup>+</sup> lymphocytes in lymph nodes draining to each one of the administration sites demonstrates the activation of cooperating T cells resulting from immunization, and it confirms the results of the HSR trial. The TCD4<sup>+</sup> lymphocytes play a very important role in effector responses, differentiated in different subsets, depending on the cytokines produced after the antigenic stimulation (Th1, Th2, Th17 and T regulatory). All these cells play a decisive role in the defense against pathogens or in the pathogenesis of several diseases. The induction and prevalence of any of these cellular patterns deserve thorough study and identification when dealing with the evaluation of a new adjuvant or vaccine immune-modulator, keeping in mind the desired effect to be obtained. In other research performed by our group, to be published soon, we demonstrated the TCD4<sup>+</sup> lymphocyte pattern of Th1 type, stimulated by trial formulations, regarding the cytokine profile elicited after immunologic activation by each formulation.

In all the groups, the values are above the negative control, which demonstrates the expansion of these subpopulations for the effector immune response.

In this research, the adjuvant properties of the new PL and Ch formulations used in this study are acknowledged to generate an immune cellular response. Other experiments, soon to be published, show the induction of specific antibodies, with a functional effector phase, which are able to produce bactericidal activity, as well as the corresponding lymphokine pattern (data not shown) and its potentiality as good vaccine candidates. The obtained results in the present research indicate, first of all, their capacity to induce a Th1 type of immune cellular

response, and to generate immunological memory mechanisms when responding to the antigenic challenge.

The Th1 type immune response is a very appropriate marker to measure the adjuvant capacity and the effectiveness of the vaccines (Stewart et al., 2006). The results of our experiments are similar to those obtained by other pre-clinical investigations, carried out by Finlay Institute, which indicate the generation of a Th1 and immunologic memory immune cellular response. Among them, the evaluation of Ch derivative from PL of *Neisseria meningitidis B* denominated (AFCo1), as powerful Th1 adjuvant in mucosa and parenteral vaccines, other studies ensure the power and safety of this adjuvant (Bracho et al., 2006; 2009). Both the PL for nasal immunization as the base material for these Ch are the original vesicles obtained by self-assembling of the external membrane proteins of *N. meningitidis B* extracted with sodium dextrocholate, as part of the productive process of the Cuban anti-meningococcal vaccine, with a mean size of 80 nm (Sierra et al., 2000). The directly obtained Ch from those PL acquire a size that can be measured in microns (Bracho et al., 2006). In this research, we have demonstrated that PL with the same antigens having a vaccine interest but obtained through other procedures and with a smaller size in the order of 10-40 nm, and Ch that derivate from them which reach a mean size below 100 nm, are also capable of inducing a Th1 response both by systemic and mucosal routes, which is very convenient given the fact that in other research we have detected productive and immune-pharmaceutical advantages in mentioned formulations.

## **Conclusion**

The capacity of the tested formulations in PL as well as in Ch types to produce a potent systemic immune response, measured by the generation of an HSR response and its immunologic consequences at the hind limb of the mouse, as well as the increase of TCD4<sup>+</sup>

lymphocytes detected, strongly suggests that all formulations evaluated in this work are able to stimulate a cellular pattern of immune response. Comparison with the well-known controls of the anti-meningococcal vaccine (Va-Mengoc-BC) helped us to better understand the potency and mechanism of immunogenicity of the new formulations tested. Despite the fact that the new formulations did not contain the known  $\text{Al(OH)}_3$  adjuvant, they were able to mount a strong Th-1 immune response equivalent to the original formulation of the vaccine. A plus benefit of a lower injection site reaction, due to lack of adjuvant gel  $\text{Al(OH)}_3$ , was observed; this has been demonstrated by our histopathological studies. Particular attention is paid to the formulation of AIF2-nCh via IN, with a Ch-like structure, which reveals superior Th-1 pattern compared to the rest of the formulations and indicates it as a possible better candidate for further studies on mucosal vaccines. Our results demonstrate that, for all the studied variants, the percentages of induration were statistically higher ( $p < 0.005$ ) compared with the negative control and become evident as vascular congestion, cellular infiltrate and other observations in histological cuts.

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455

456    **Conflicts of interest:** The authors declare no conflict of interest.

457    **Contribution:** All authors had equal contribution to this paper.

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536

## List of figures

**Figure 1.** Electron Microscopy Micrographs **A**, **A1** and **C**: Transmission (TEM), Microscope PHILIPS CM-12, magnification: 500,000X and voltage: 60kV. Micrograph **B**: Scanning Electron Microscope (SEM) LEO-440 VP, magnification: 100,000X. Micrographs **A**, **A1** and **B**: nanometric structures are observed with the shape of liposomes. Micrograph **C** nanometric structures with the shape of cochleates. The numbers inserted in figure indicate the size of the structures.

**Figure 2.** Footpad inflammation. The footpad inflammation of the left posterior extremity in comparison with the right one is shown.

**Figure 3.** Delayed Type Hypersensitivity Reaction (DTH-R) evaluated by the differential swelling of the footpad of both posterior extremities of Balb/c mice. The magnitude of the DTH-R was measured at 21 days after the last immunization dose; challenging intradermally with 15µg PD in the left posterior footpad, (PBS) phosphate buffered saline was injected as a control in the right footpad of each animal. The magnitude of the inflammation in both extremities was measured 48 hours after the challenge using an electronic caliper. The magnitude of the induced DTH-R has been presented as the percentage of mean inflammation of the extremity  $\pm$  Standard Deviation of the Mean (%IP  $\pm$  MSD). A) Experiment on intramuscular route of inoculation. B) Experiment on intranasal route of inoculation. Every value represents the mean of five determinations  $\pm$  Mean Standard Deviation. Different symbols indicate statistically significant differences with \*p<0.05 (ANOVA+ Duncan Test).

559 **Figure 4.** Microphotographs of histological sections of footpad of posterior extremities. **Ed:**  
560 Right posterior extremity, control inoculated with PBS. **Ei:** Left posterior extremity  
561 inoculated with PD antigen. **A:** grade +; **B:** grade ++; **C:** grade +++; **D:** grade ++++ (HE  
562 150X). White ovals indicate zones of inflammatory cell accumulation.

563 **Figure 5.** Representative histograms from the data analysis using the CellQuest (CF) program  
564 for the quantification of TCD4<sup>+</sup> lymphocytes in terms of percentage of the total cell counting  
565 in sub-inguinal lymph nodes of the different experimental groups A(AIF1-nPL), C(AIF1-  
566 nCh), E(AIF2-nPL), G(AIF2-nCh), I(PD-Tp), ST(VA-MENGOC-BC®) and CNR1 (Control  
567 Group immunized with PBS) by intramuscular route. The specific labeling was performed  
568 with the anti CD4<sup>+</sup> PE-Cy5 monoclonal antibody. The gray histogram represents the non-  
569 labeled cells; the red line represents cells labeled with the specific antibody. Range of values  
570 of not immunized animals: 50-60% of TCD4<sup>+</sup> lymphocytes.

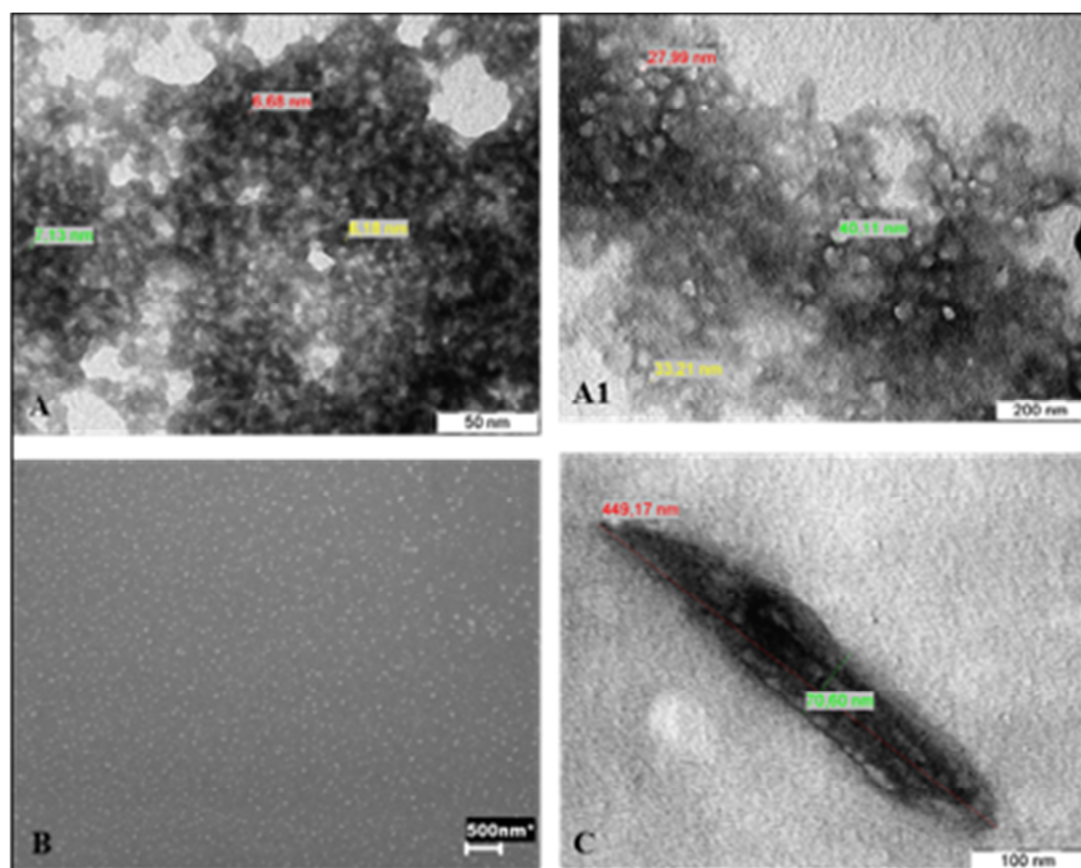


Fig. 1



Fig. 2

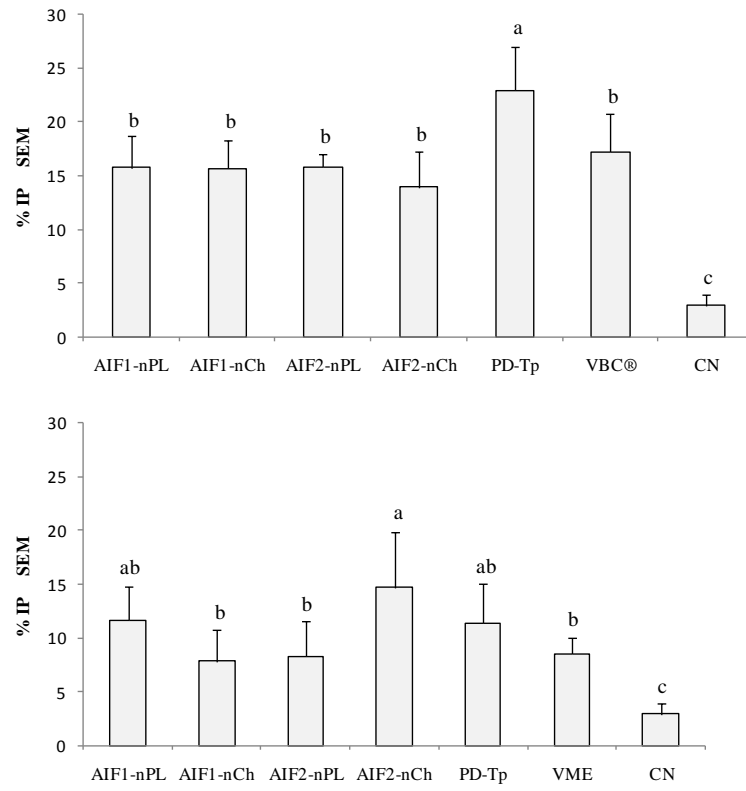


Fig. 3

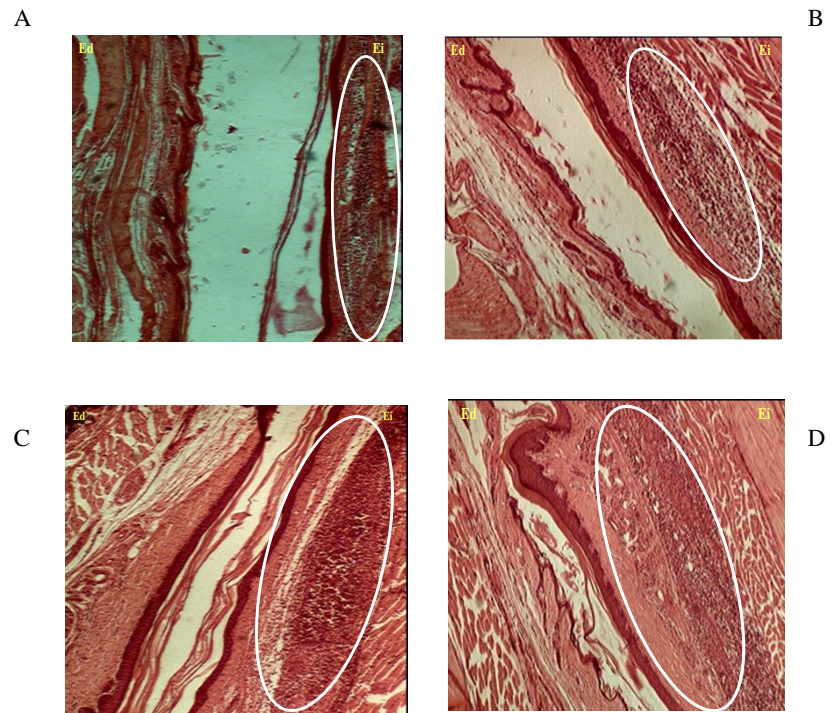


Fig. 4

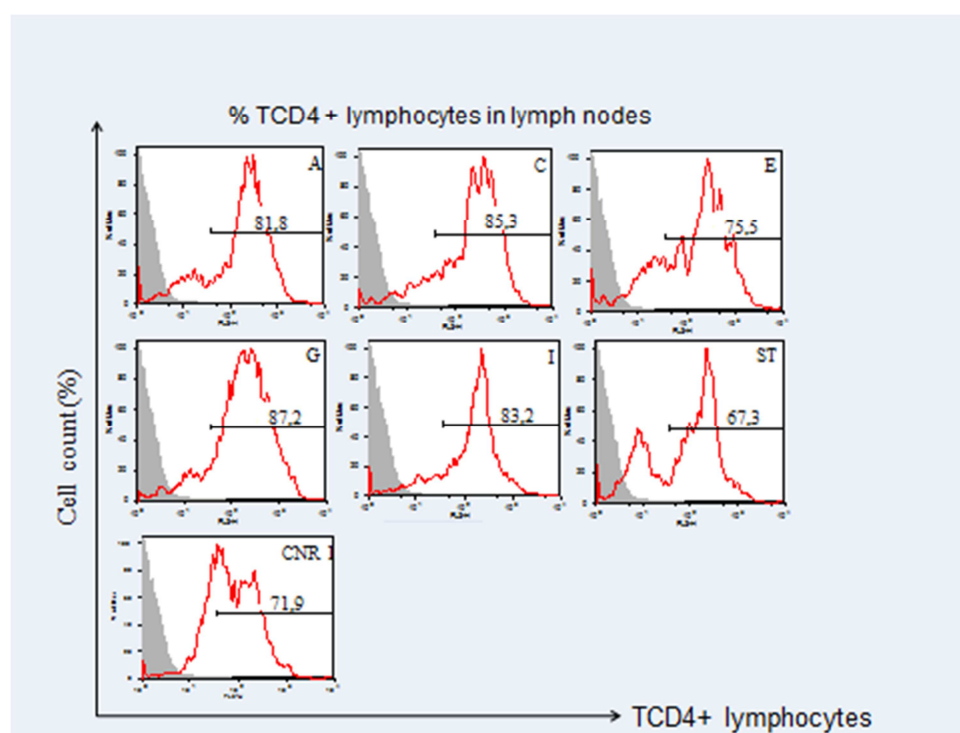


Fig. 5



**Table I.** Delayed Type Hypersensitivity Assay. Classification by inflammation degree of the DTH-R observed in the different experimental groups, based on the degree of inflammation and infiltration observed according to histopathology studies of foot pad lesions

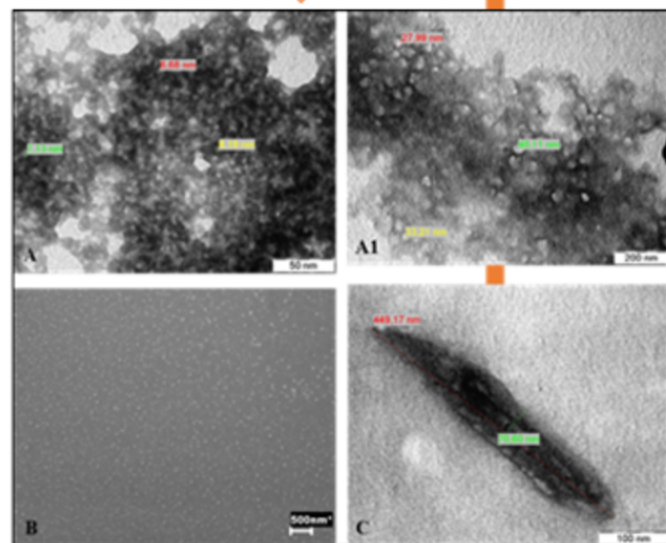
Formulations		AIF1-nPL	AIF1-nCh	AIF2-nCh	AIF2-nCh	PD-Tp	VBC®:	VME
ROUTE	IM	+++	+++	+++	+++	++++	+++	-
	IN	+++	++	++	+++	+++	-	+

+: slight; ++: moderate; +++: strong; ++++: serious.

VBC®: Cuban antimeningococcal Vaccine VA-MENGOC- BC®.

VME: Outer Membrane Vesicles from *Neisseria meningitides Group B*.

New adjuvant formulations  
Proteoliposomes <40nm  
Cochleates <100nm

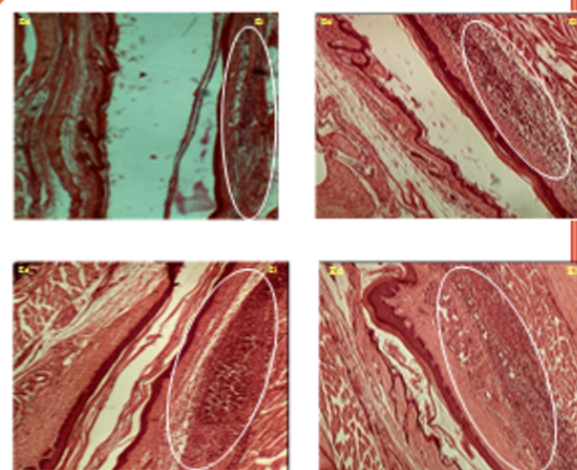


Immune cellular response  
type Th1  
Delayed-Type  
Hypersensitivity assay

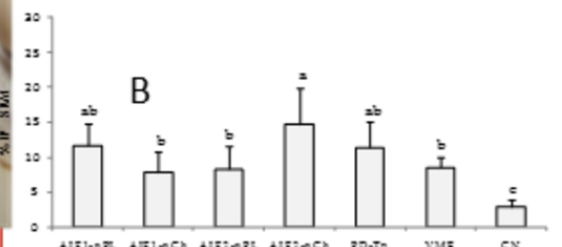
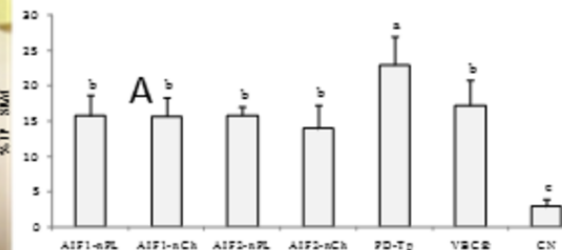
*Neisseria meningitidis B*



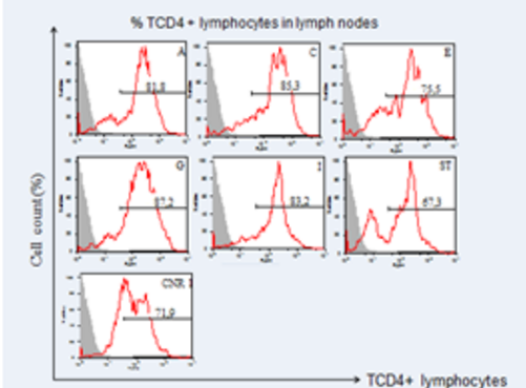
Feet pat inflammation



Histological sections of foot pad of posterior extremities



Delayed Type Hypersensitivity Reaction



T CD4<sup>+</sup> Lymphocytes

All formulations in the trial stimulate type-Th1 cellular immune response, similar to positive control, which corroborates the potentiality of the formulations as vaccine adjuvants.