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

3

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22

23 Abstract

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

44

45 **Key words:** Delayed-Type Hypersensitivity; Nanoproteoliposomes; Nanocochleates;
46 adjuvant; anti-meningococcal vaccine B; TCD4⁺.

47 **1. Introduction**

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

52 The Cuban anti-meningococcal vaccine VA-MENGOC-BC® has been one of the most
53 efficient approaches to protect against B and C meningococci, with over 60 million doses
54 administered in humans (Vermikos and Medini, 2014). This vaccine is formed by
55 proteoliposomes (PL) derived from the *Neisseria meningitidis* serogroup B (NMB) external
56 membrane, which are the main antigenic component, and the purified capsular polysaccharide
57 of serogroup C, whose components are absorbed in aluminum hydroxide gel (Sierra et al.,
58 1991; 2000). The latter has been a broadly used and approved adjuvant for more than one
59 century in human vaccines. However, its immuno-potentiating capacity directs the immune
60 response toward a predominating type-Th2 pattern, and it is useful only in parenteral
61 applications, fundamentally by the intramuscular route (Pérez et al., 2007). It has
62 demonstrated in general a good safety record and strong antibody induction, but because it is
63 not suitable for inducing optimal type-Th1 or cytotoxic (CTL) responses, its use is appropriate
64 when the antibody systemic response is sufficient to provide immunity (O'Hagan and
65 Valiante, 2003) but inappropriate when a potent type-Th1 or CTL response is required to
66 provide immune protection. Another of its disadvantages is reactogenicity due to the
67 formation of granulomas resulting from the persistence of alumina in the injection site
68 (Kurella et al., 2000). Studies focusing on vaccines show the paramount importance of the
69 connection between immunogenicity and reactogenicity (Bracho et al., 2006; Girard et al.,
70 2007; Lastre et al., 2006). For this reason, particulate vaccine formulations have been
71 designed which do not require the additional use of this type of aluminum-derived adjuvant.

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

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

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

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

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

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

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

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

133 **2. Materials and methods**

134 **2.1. Adjuvant formulations**

135 The adjuvant formulations were designed using, as an antigen, a complex of NMB proteins
136 extracted from bacteria (the extraction was carried out using a mild DOC detergent method
137 and the detergent was removed with ethanol; the latter was in turn removed using the gel
138 filtration method) (Bracho et al, 2006) and purified without forming vesicular structures,
139 which in this article are identified as differentiated proteins (PD). They are combined with
140 immune-modulating components of a defined molecular structure, to produce two formulation
141 groups (1 and 2) that are structured in neo-proteoliposome (nPL) and neo-cochleate (nCh)
142 forms. The obtained formulations were named AIF1-nPL, AIF1-nCh, AIF2-nPL and AIF2-
143 nCh, (AIF: Adjuvant IFAL – “Finlay”). Liposomes were developed starting from a 1.2-
144 dioleoylphosphatidylserine (DOPS) mixture and cholesterol (Sigma Aldrich Co.), using the
145 procedure of dehydration-rehydration (Kirby and Gregoriadis, 1984). The Ch were obtained

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

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

- 155 1. Type: PL as immunomodulator: AIF1nPL- 15µg protein PD (VME) in 25 µL buffer
156 Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as
157 detergent, pH 7.4 + integral LPS 1%.
- 158 2. Type: cochleate as immunomodulator: AIF1nCh-5µg protein PD (VME) in 25 µL
159 buffer Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG)
160 SIGMA® as detergent, pH 7.4 + integral LPS 1%.
- 161 3. Type PL as immunomodulator: AIF1nPL- 15µg protein PD (VME) in 25 µL buffer
162 Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as
163 detergent, pH 7.4, without integral LPS.
- 164 4. Type cochleate as immunomodulator: AIF2nCh-15µg protein PD (VME) in 25 µL
165 buffer Tris 2 mM, NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG)
166 SIGMA® as detergent, pH 7.4, without integral LPS.
- 167 5. Type Control (soluble): PD-Tp-15 µg protein PD (VME) in 2 5µL buffer Tris 2 mM,
168 NaCL 150 mM, n-Octyl-β-D-Thyoglucoipiranoside (nOG) SIGMA® as detergent, pH
169 7.4, without integral LPS and without forming cochleate or PL.

170 6. Type Control for intra muscular applications: VBC-Va-Mengoc-BC (Bracho et al,
171 2006).

172 7. Type Control for intra nasal applications: VME-Va-Mengoc-BC, as recommended by
173 Bracho et al, 2006, but without Al(OH)₃ gel.

174 **2.2. Characterization of the nanoproteoliposomes and nanocochleates using electronic** 175 **microscopy**

176 *Electronic Transmission Microscopy*

177 The electronic images were obtained by Electronic Transmission Microscopy with a CM12
178 Phillips microscope. The samples were processed by negative staining using the traditional
179 drop method. After the adsorption of 5 µL of the nPL and nCh formulations on a grill covered
180 with coal, the samples were tinted with phosphotungstic acid at 2% and observed at 100,000X
181 magnification.

182 *Scanning electron microscopy*

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

187 **2.3. Animals used in the experiment**

188 The number of animals (BALB/c mice) required was calculated before the study, taking into
189 account all the tests we were considering at that time; the mice were brought from the Center
190 for the Production of Laboratory Animals, Cuba (CENPALAB, breeding-production facility)
191 and had approximately identical characteristics in terms of isogenic origin, sex (males), age
192 (6-8 week old) and, implicitly, weight, etc. To assure complete homogeneity, random lists

193 were generated, with the formulations assigned per animal according to the order of extraction
194 of each animal from the cages. Care to mice was assured, according to established
195 institutional norms set in the Guide for the Care and Use of Laboratory Animals, 8th Edition,
196 2011, from the Institute for Laboratory Animal Research, Division on Earth and Life Studies,
197 National Research Council, of the National Academies (<http://www.nap.edu>) under free-of-
198 pathogen micro isolation conditions. These experiments were carried out with authorization
199 and approval from the Ethics Committee of Laboratory Animals of CENPALAB. The design,
200 implementation and evaluation of research that involved using experimentation animals
201 abided by the general bioethics principles of refinement, replacement and reduction (RRR), as
202 well as by laboratory and laboratory-animal-handling good practices (Hernández, 1997).

203 **2.4. Study description**

204 A total of 65 animals were used, which were randomly distributed into 13 groups (5 animals
205 per group). Formulations were administered both by the intramuscular (IM) as well as
206 intranasal (IN) immunization routes. The IM immunization schedule consisted of two
207 inoculations, the first one at the beginning of the study and the second after 14 days. A 15 µg
208 dose corresponding to the protein concentration was administered in 250 µL of saline
209 phosphate buffer (PBS). Mice were inoculated using a deep intramuscular puncture in the left
210 quadriceps of the hind limb. The IN immunization consisted of three inoculations, with
211 intervals of 7 days. The animals received a 15 µg dose corresponding to the protein
212 concentration in 25 µL of PBS (12.5 µL in each nasal fossa). Each experimental group was
213 immunized with a trial formulation. The VA-MENGOC-BC® vaccine was IM administered
214 (25 µg/ dose), while the VME of NMB was IN administered (15 µg/ dose), both as controls. A
215 negative control group was also included to which PBS was administered; it is identified as
216 CN.

217 **2.5. Delayed-type hypersensitivity trial**

218 After 21 days, all the animals received, by the intradermal immunization route, 15 µg of PD in
219 30 µL of PBS (0.5 mg/mL in PBS) in the left hind footpad, and 30 µL of PBS in the right one,
220 the latter used as control. The absence of inflammatory reaction at 4 and 24 hours was
221 confirmed. The width of the paws was measured in millimeters (mm) using a digital Vernier
222 caliper, 0-150 mm (OMNI - TECH), between the third and fourth pulp-protuberances of both
223 hind limbs, in order to measure the inflammation produced at 48 hours. The percent of
224 induced inflammation (%IP), as a criterion of HSR reaction, was calculated according to the
225 bellow formula (Lincopan et al., 2009), with results expressed as % IP± the standard error of
226 the mean (SEM).

227

228 $\% \text{ IP} = 100[(\text{left hind footpad thickness}) - (\text{right hind footpad thickness})] / (\text{mean thickness un-}$
229 $\text{injected left hind footpad}).$

230

231 **2.6. Histological study of hind extremities**

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

239 **2.7. TCD4⁺ Lymphocytes analysis by flow cytometry**

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

246 **2.8. Statistical analysis**

247 The statistical processing of the results was performed using the Statistica program, version
248 6.0, on Windows. The Kolmogorov-Smirnov test was used for assessing the normality of the
249 distribution and the Levene test used to evaluate the homogeneity variance. A one-way
250 variance analysis was used (ANOVA) by means of a Dunnet and Duncan test. In all the cases
251 a $p \leq 0.05$ significance level was used.

252 **3. Results**

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

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

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

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

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

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

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

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

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

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

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

318 **4. Discussion**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

427 **Conclusion**

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

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

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455

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

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535 proteoliposome derived Cochleate production. *Vaccine* 2006; 24(2): 94–95.

536

537 **List of figures**

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

544

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

547

548 **Figure 3.** Delayed Type Hypersensitivity Reaction (DTH-R) evaluated by the differential
549 swelling of the footpad of both posterior extremities of Balb/c mice. The magnitude of the
550 DTH-R was measured at 21 days after the last immunization dose; challenging intradermally
551 with 15 μ g PD in the left posterior footpad, (PBS) phosphate buffered saline was injected as a
552 control in the right footpad of each animal. The magnitude of the inflammation in both
553 extremities was measured 48 hours after the challenge using an electronic caliper. The
554 magnitude of the induced DTH-R has been presented as the percentage of mean inflammation
555 of the extremity \pm Standard Deviation of the Mean (%IP \pm MSD). A) Experiment on
556 intramuscular route of inoculation. B) Experiment on intranasal route of inoculation. Every
557 value represents the mean of five determinations \pm Mean Standard Deviation. Different
558 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⁺ 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⁺ 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⁺ lymphocytes.

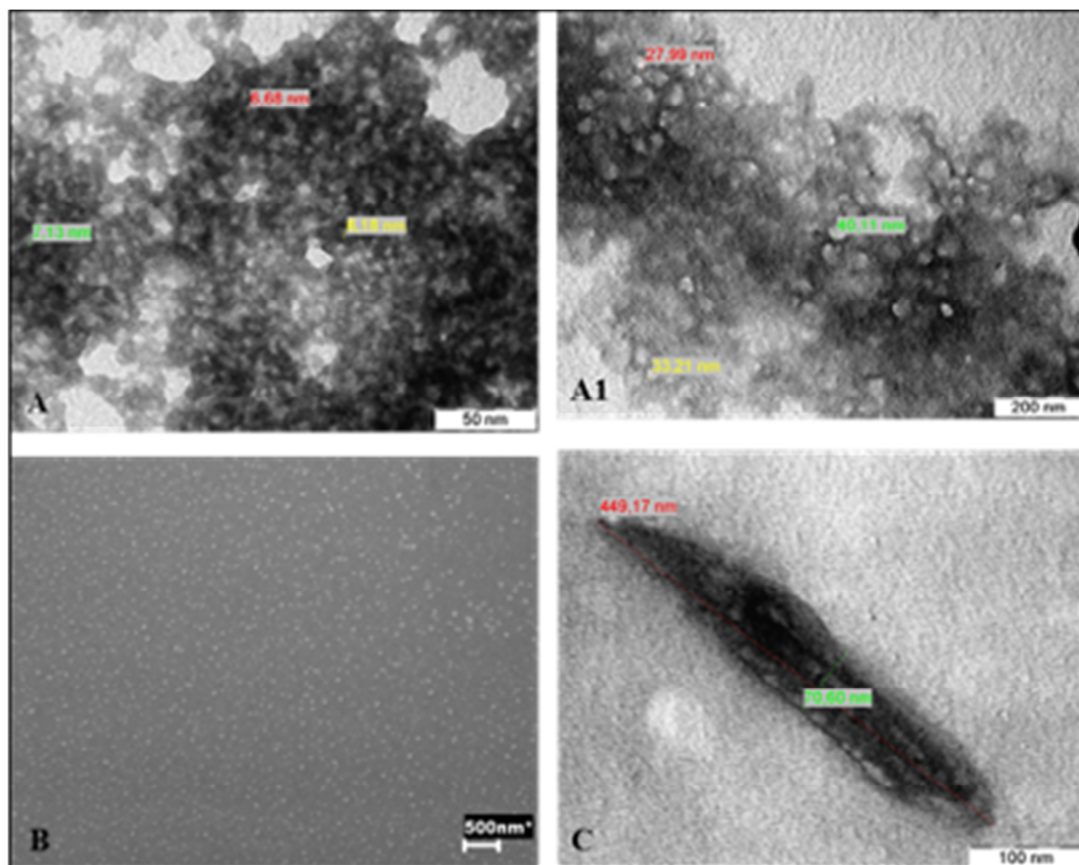


Fig. 1

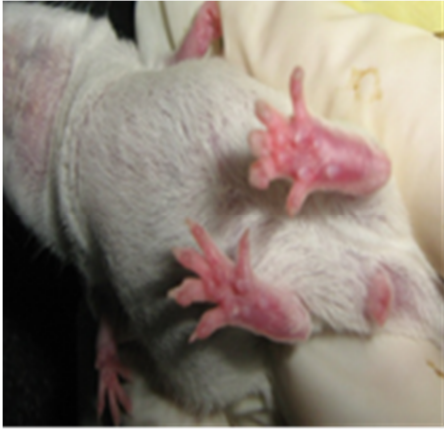


Fig. 2

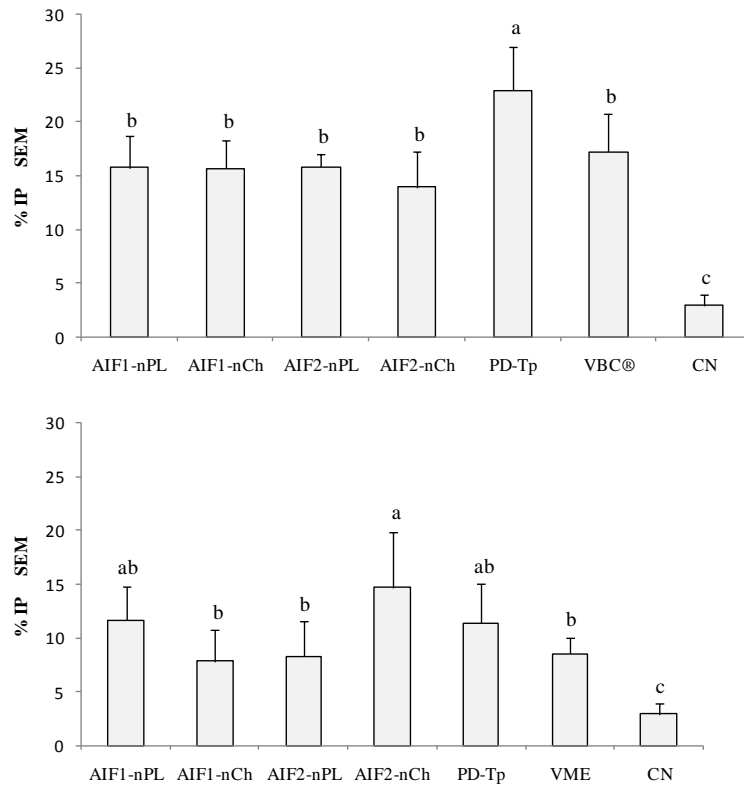


Fig. 3

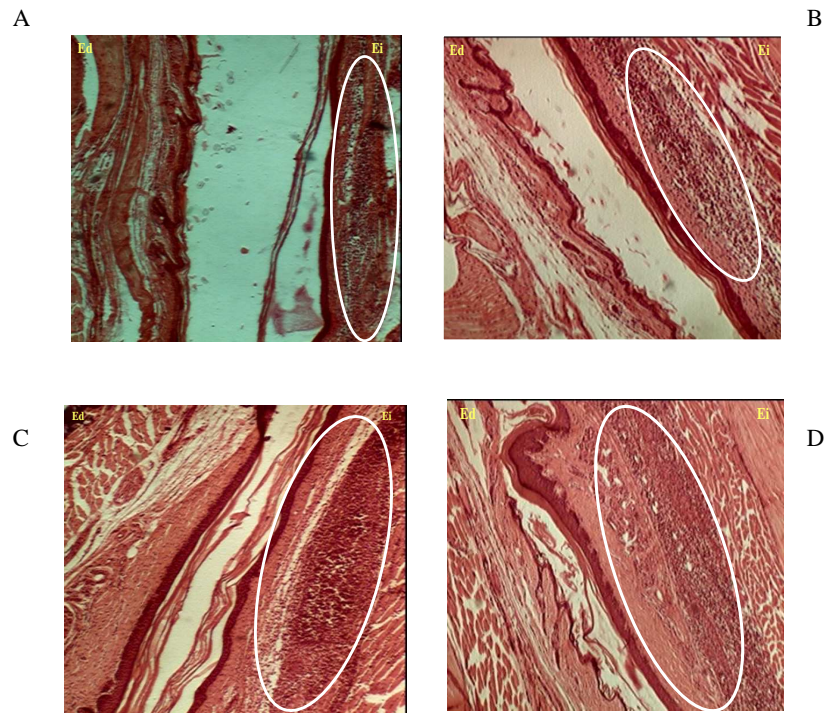


Fig. 4

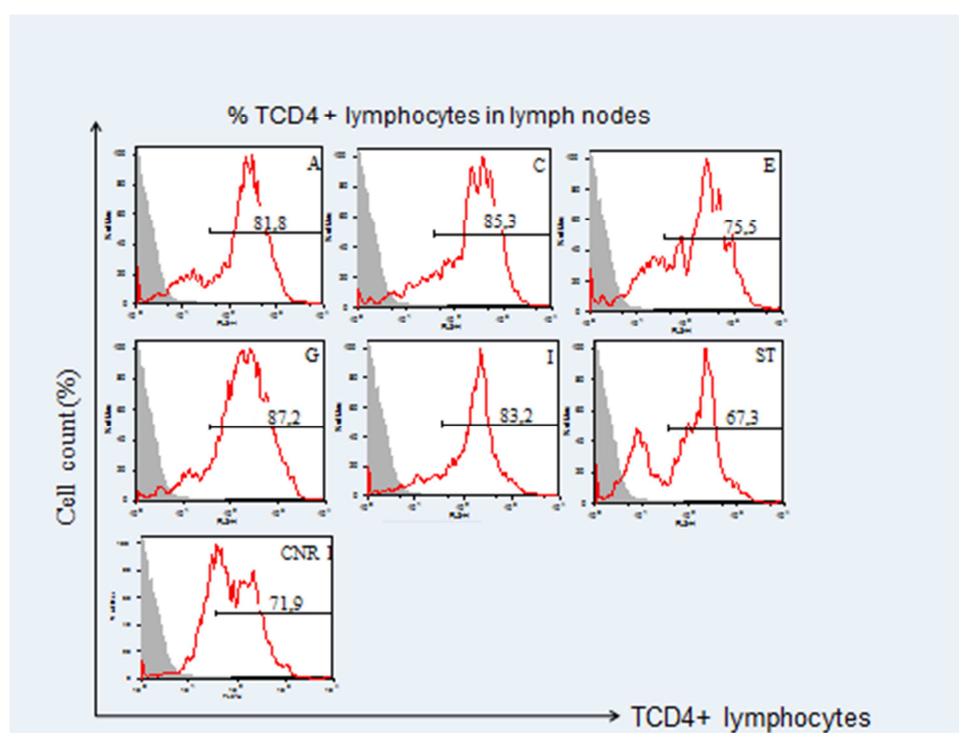


Fig. 5

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

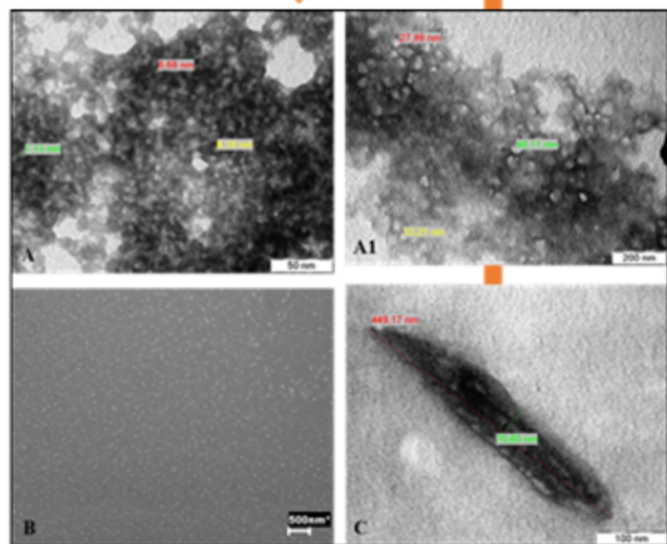
Formulations		AIF1-nPL	AIF1-nCh	AIF2-nCh	AIF2-nCh	PD-Tp	VBC®:	VME
ROUTE	IM	+++	+++	+++	+++	++++	+++	-
	IN	+++	++	++	+++	+++	-	+

+: 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

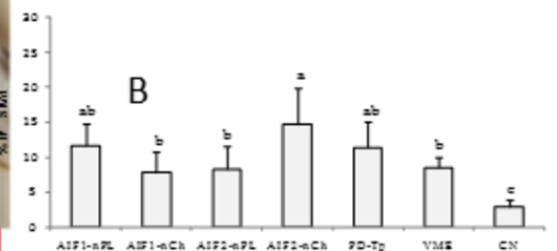
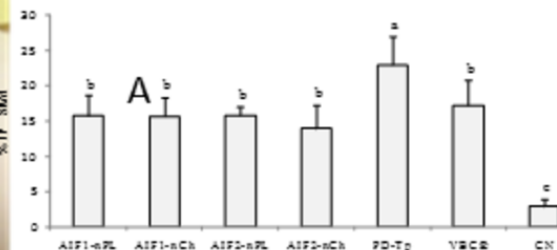


Immune cellular response
 type Th1
 Delayed-Type
 Hypersensitivity assay

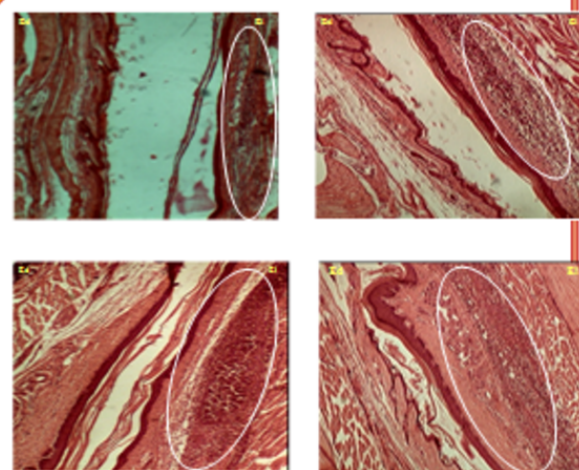
Neisseria meningitidis B



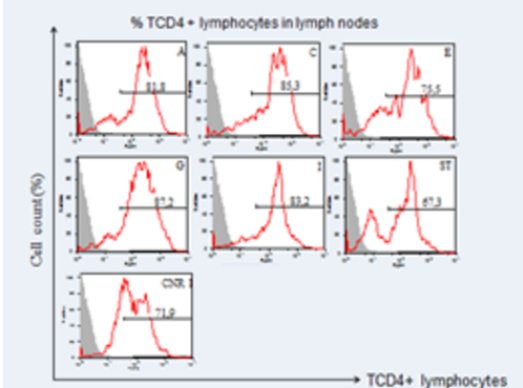
Feet pat inflammation



Delayed Type Hypersensitivity
 Reaction



Histological sections of foot
 pad of posterior extremities



T CD4⁺ 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.