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Assessment of repeat dose toxicity of Cochleate derived from *Neisseria meningitidis* proteoliposome in Sprague Dawley rats

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The AFCo1 cochleate is a potential novel adjuvant derived from *Neisseria meningitidis* B proteoliposome. The aim of this study was to assess the safety of AFCo1 by repeated doses in Sprague Dawley rats. Rats were grouped for treatment with AFCo1, placebo formulation or control. Four similar doses of the test substance were instilled every five days. Intranasal dose of 100 μL was used, and the body weight, water and food intakes were monitored as well as the clinical symptoms. Rats were sacrificed at 3, 14 and 28 days after the last inoculation and anatomopathological studies were conducted. Clinical observations were carried out for the study and a number of rats from each group were sacrificed 3 and 14 days after the last dose in order to conduct hematological, hemochemical and anatomopathological studies. Clinical symptoms, food and water intakes, and body weight did not show differences of toxicological relevance. The histological changes found were mild and similar in the three groups. AFCo1 is potentially safe by nasal route for human use as evidenced by the absence of local and systemic signs of toxicity in Sprague Dawley rats.

Keywords: *Neisseria meningitidis*, Sprague Dawley, repeated dose, proteoliposome.

Introduction

The mucosal immune system has been recognized as the first defense of the host against pathogens entering the gastrointestinal or the upper respiratory tracts (1). Mucosal immunization has been well documented and it is a highly effective way to stimulate local and systemic immune responses.

Local specific IgA response, systemic specific IgG response and cell-mediated immunity have been induced by mucosal immunization (2, 3). However, a close relationship between the adjuvants used and the immune response elicited regarding different antigens has been demonstrated (4). Thus, the development of new vaccines has increased the need for new and more powerful adjuvants.

Finlay Institute has developed a group of adjuvants, from which the AFCo1 cochleate has resulted particularly effective by both mucosal and parenteral routes (5,6).

The intranasal administration of adjuvants has been poorly explored from the toxicological point of view. Since only one layer of cells separates the lumen of the nasal cavity from a rich vascular net in the *lamina propria* raises safety concerns.

That is why; the objective of the present paper was to perform repeated doses safety studies of AFCo1 by intranasal administration in Sprague Dawley rats.

Materials and Methods

Test substance

The active ingredient of AFCo1 is a cochleate structure derived from the proteoliposome of *Neisseria meningitidis* serogroup B with a protein content of 1 mg/mL. Every 100 μL also contains sodium chloride (292 μg), Tris (360 μg), calcium chloride (73.5 μg), thiomersal (10 μg) and water for injection as solvent**.** The placebo formulation contained only the auxiliary components detailed above.

Animal model

Male and female, 5-6 weeks old, 150-200 g body weight, Sprague Dawley rats supplied by the National Center for the Production of Laboratory Animals (CENPALAB, Cuba) were used. Five rats were housed per each T4 (floor area: 1800 cm^2) polycarbonate cage (Tecniplast, Italy). Sugar cane bagasse sterilized in autoclave (15 min, 121 ^{o}C) and changed twice a week was used as bedding.

Pelleted food, produced by CENPALAB, and fresh drinking water were administered for *ad libitum* consumption.

Room temperature (22-25 °C), relative humidity (60-65%) and light cycle (10 h light-14 h dark) were controlled and recorded twice a day. Animals were sacrificed by intraperitoneal overdose of pentobarbital (100 mg/kg).The protocol of the study was approved by the Ethics Committee for the Care

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and Use of Laboratory Animals from Finlay Institute and it is in accordance with international standards on the topic (7, 8).

Repeated dose toxicity test. Four doses of 100 μL each were nasally instilled every 5 days. The schedule proposed for human use conceives only there doses, but a further dose was administered to the rats in order to maximize their exposition to the test substance. Animal housing and maintenance as well as the experimental groups were as described for the single dose toxicity study. Similarly, clinical symptoms, food and water intakes, and body weight were studied. Additionally, hematology, blood biochemistry, and anatomopathological studies were conducted on groups of rats sacrificed 3, 14 and 28 days after the last inoculation.

Hematology included quantification of hemoglobin, hematocrit, leukocytes, differential count of leukocytes, count of erythrocytes and platelets. Blood serum chemistry measured the levels of glucose, urea, creatinine, alkaline phosphatase, total proteins, triglycerides, cholesterol, direct and total bilirubin (BIL-D BIL-T), creatine phosphokinase, transaminases and urates. All the hemochemical determinations were carried out using commercial kits (CENTIS, Havana, Cuba) following the procedures recommended by the producer.

The anatomopathological studies included the necropsy of all the rats and tissue sampling for histological studies. The relative weight of the heart, thymus, lungs, kidneys, liver, and spleen was calculated. The irritation index caused by the product on the nasal mucosa was also calculated.

Statistical Analysis

The statistical package STATISTICA 6 (StatSoft, Inc. (2003). Statistica - data analysis software system, version 6. www.statsoft.com) was used. P values under 0.05 were considered significant. Body weights were compared by repeated measures analysis of covariance, using values at the beginning of the assay as covariate. The normality and homogeneity of variance assumptions were tested by means of Shapiro-Wilk's W test and Levene's test, respectively, in order to decide whether conducting parametric (Analysis of variance and least significant difference tests) or nonparametric procedures (Kruskal-Wallis and distribution-free multiple comparisons tests). Finally, the proportion of histological changes was compared by log-lineal analysis.

Results and Discussions

Clinical symptoms, food and water intakes, and body weight did not show differences of toxicological relevance.

Anatomopathological, hematological and hemochemical analysis performed 72 h after the last inoculation intended to reveal early side effects while those at 14 days were planed to assess recovery of damages or long-term effects (9).

Hematology and blood chemistry analysis did not show statistical differences among experimental groups (Table 1). Relative organ weights were also statistically similar (data not shown), with the exception of the right kidney weight in females that received AFCo1 and placebo that were higher ($p<0,05$) to that of control rats. However, no histological changes were found in the kidneys of such animals that could support an association with toxic effects.

Petechial hemorrhages in cervical lymph nodes in rats regardless of the sex, treatment group or sacrifice time were found at necropsy again. Due to their unspecific presentation, they were associated to restraint during sedation and bleeding. Histology (Table 2) revealed inflammatory cells infiltrating the nasal cavity. Mononuclear cells and sporadic suppurated focuses were also found at mucosal and submucosal tissues of the nasal septum as well as dorsal and ventral nasal cornets. Besides, nasal congestion, edema and degeneration of the superficial epithelium were found. However, statistical analysis did not evidence differences ($p>0,05$) among the histological changes in the three experimental groups.

A relevant animal model for the toxicological assessment of a vaccine or adjuvant must mount an immune response as expected to happen in the target species.

Thus, intrinsic toxicity due to the constituents, contaminants, the interaction among them and that related to the immune response elicited by the test product could be evaluated (9, 10). Sprague Dawley is an outbred rat strain preferred for preclinical safety studies due to their heterogeneous response (7); furthermore, a pilot study demonstrated that they respond immunologically to AFCo1 after nasal instillation. Therefore, that strain was considered a relevant biomodel for the safety assessment of AFCo1.

In order to increase rat exposure to AFCo1 and to test the potential risk of accidental overdosing, a four-dose toxicity study was carried out. This design is in correspondence with current trends in the preclinical evaluation of preventive vaccines that suggest the administration of a further dose to that proposed for the clinical use (11).

The growth curve, water and food intakes, blood chemistry values and the relative organ weights of the rats in both tests conducted agreed with those observed for Sprague Dawley rats during the preclinical assessment of other candidate vaccines in our facilities (12-15) and reference values reported elsewhere (16).

The results of the hematological exam showed that the hemoglobin (Hb-g/l), red blood cell (GR-106/mm^3) and total lymphocytes (LT -103/mm^3), not differed statistically among treated and control groups, also this results was similarly to the normal values for the rats line used (Table 1).

Table 1. Hematology and Serum Blood Chemistry of rats under repeated dosing. Means and 95% confidence intervals (in brackets). P values refer to ANOVA comparisons among treatment groups.

Variable	Females			Males			P
	AFCo1	Placebo	Control	AFCo1	Placebo	Control	
Hb (g/l)	136 (126-146)	139 (129-150)	135 (125-145)	144 (134-154)	134 (124-144)	147 (137-158)	0.64
GR (10^6/mm^3)	6.8 (6.2-7.4)	6.1 (5.5-6.7)	6.3 (5.7-6.9)	7.0 (6.4-7.6)	7.2 (6.6-7.8)	6.8 (6.2-7.4)	0.46
LT (10^3/mm^3)	6.1 (5.0-7.2)	7.1 (6.0-8.2)	7.0 (5.9-8.1)	8.5 (7.4-9.6)	8.2 (7.1-9.3)	7.6 (6.5-8.7)	0.79
Glucose (mmol/L)	5.2 (4.4-6.0)	5.0 (4.2-5.7)	5.4 (4.7-6.2)	5.9 (5.1-6.6)	5.5 (4.7-6.2)	6.3 (5.5-7.0)	0.22
Creatinine (μmol/L)	47.2 (43-51)	47.8 (43-52)	51.5 (47-56)	42.0 (38-46)	45.4 (41-49)	45.4 (41-49)	0.17
TGO (U/L)	245.3 (221-269)	241.9 (218-265)	182.5 (159-206)	180.5 (157-204)	179.5 (156-203)	193.4 (170-217)	0.07
TGP (U/L)	56.2 (51-61)	51.4 (46-56)	48.1 (43-53)	51.1 (46-56)	55.9 (51-61)	53.2 (48-58)	0.32
ALP (U/L)	256.6 (219-222)	257.7 (221-294)	283.3 (247-320)	372.9 (336-409)	335.1 (298-371)	364.5 (328-401)	0.31
Total Bilirubin (mg/dL)	4.2 (3.3-5.1)	4.0 (3.2-4.8)	3.4 (2.5-4.2)	3.7 (2.9-4.5)	4.5 (3.7-5.3)	3.6 (2.8-4.4)	0.19
Total Proteins (g/dL)	58.7 (56-61)	55.4 (53-57)	56.7 (54-59)	49.7 (47-52)	50.0 (48-52)	51.8 (50-54)	0.17
Urates (μmol/L)	74.7 (61-89)	78.0 (63-93)	70.1 (56-84)	52.9 (39-67)	71.0 (57-85)	47.5 (33-62)	0.09
Cholesterol (mmol/L)	1.5 (1.4-1.6)	1.5 (1.4-1.6)	1.6 (1.5-1.7)	1.2 (11-1.3)	1.3 (12-1.4)	1.3 (1.2-1.4)	0.55
Triglycerides (mmol/L)	0.69 (0.5-0.8)	0.79 (0.6-0.9)	0.73 (0.6-0.9)	0.64 (0.5-0.8)	0.59 (0.4-0.7)	0.55 (0.4-0.7)	0.79
CPK (U/L)	2817 (2488-3145)	2419 (2099-2738)	2363 (2043-2683)	2027 (1707-2347)	2484 (2168-2804)	2492 (2172-2812)	0.98
Urea (mmol/L)	7.4 (7.0-7.9)	6.7 (6.2-7.1)	6.6 (6.2-7.0)	5.4 (5.0-5.9)	5.7 (5.2-6.1)	5.6 (5.2-6.1)	0.34

Hemoglin (HB), Red Blood Cell (GR), Total Lymphocytes (LT), Glutamic Oxaliacetic Transaminase (TGO), GLUTAMIC pIRUVIC tRANSAMINASE (TGP), ALKALINE PHOSPHATASE (ALP), CREATININ PHOSPHOKINASE (CPK).

Histological changes were either part of physiologic reactions or incidental, but a connection with toxicity could not be established. Mucosal vascular congestion found reflects the normal physiologic hyperemia of the nasal epithelium (17). Focally aggregated or diffusely distributed lymphoid cells found at the *lamina propria* are part of the functional anatomy of mucosal tissues and are mainly represented by T CD4+ and B lymphocytes (18).

The apoptotic lymphocytes and secondary follicles in the cortical and paracortical area of lymph nodes (Fig. 1 and Fig. 2), have also been observed by other authors and are associated to the response of secondary lymphoid tissues to the antigenic stimulus (18, 19).

Finally, focal interstitial pneumonia is often observed as a consequence of viral infections in rats mainly found in animals like these which are maintained in a conventional sanitary environment (20-22). However, it is interesting that rats treated with AFCo1 developed interstitial pneumonia in a lower proportion ($p<0,05$) compared to placebo and control rats. That could probably be related to the immunomodulating effect of AFCo1 (5, 6).

Nasal instillation of AFCo1 in Sprague Dawley rats at a high dose and frequency compared to the schedule proposed for human use was neither local nor systemically toxic. Taking evidenced presented here and previous pharmacology and toxicity tests conducted in to account, AFCo1, the cochleate derived from *Neisseria meningitidis* proteoliposome is considered potentially safe for human use.

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Table 2. Repeated dose toxicity test: frequency of lesions in rats 3 and 14 days after the last dose.

Lesions	3 days/14 days						
	Placebo	Control	AFCo 1	Placebo	Control	AFCo 1	P
Inflammatory infiltrate in nasal cavity	0/10	3/10	3/10	2/10	1/10	3/10	0.35
Secondary follicles in regional lymph nodes of the digestive system	3/10	4/10	4/10	5/10	2/10	5/10	0.64
Focal interstitial pneumonia	1/10	3/10	0/10	7/10	6/10	2/10	0.04
Mild suppurated focuses in liver	1/5	0/5	0/5	0/5	0/5	0/5	0.77
Suppuration focuses in lung parenchyma	4/5	0/5	0/5	0/5	0/5	0/5	0.07
Mild hepatic degeneration	1/5	0/5	1/5	0/5	0/5	0/5	0.78
Mild suppurated focuses in lung	0/5	0/5	1/5	0/5	0/5	0/5	0.77
Perivascular infiltrate of mononuclear cells in the lung	0/5	1/5	0/5	1/5	1/5	2/5	0.85

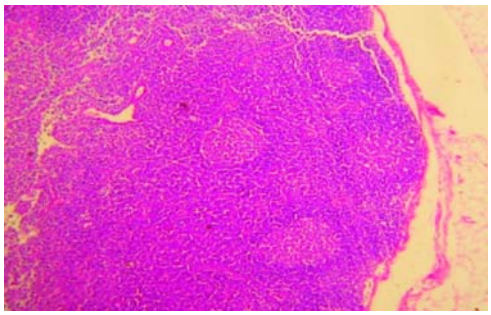

Fig. 1. Lymph node of female placebo rat in the repeated dose toxicity test sacrificed 3 days after the last inoculation. Secondary follicles in the subcortical and paracortical areas (arrows) were observed. H.E 120X.

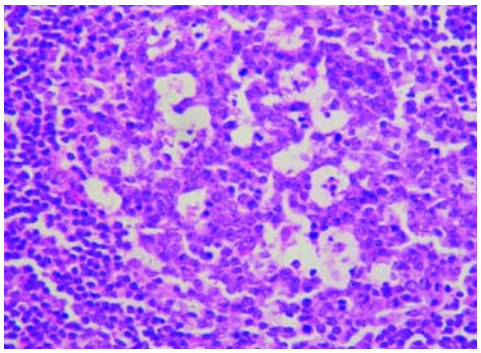

Fig. 2. Secondary follicle with apoptotic lymphocytes (arrows) in the regional lymph node, single dose toxicity test, female rat inoculated with AFCo1. H.E 220X.

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Evaluación de la toxicidad por dosis repetida de un cocleato derivado de un proteoliposoma de *Neisseria meningitidis* en ratas Sprague Dawley

Resumen

El cocleato AFCo1, derivado de un proteoliposoma de *Neisseria meningitidis* B, es un nuevo y potente adyuvante vacunal. En el presente trabajo se evaluó la seguridad del AFCo1 mediante un estudio de dosis repetida en ratas Sprague Dawley. Los animales se agruparon en: tratados con AFCo1, placebo y control. Se les administraron cuatro dosis de 100 μL durante 5 días por vía intranasal. Se monitoreo el peso corporal, consumo de agua y alimento y los síntomas clínicos; así como estudios hematológicos y bioquímicos. Las ratas se sacrificaron a los 3, 14 y 28 días después de la última inoculación y se les realizó, además, pruebas anatomopatológicas. Los síntomas clínicos, el consumo de agua y alimento y el peso corporal no mostraron diferencias de relevancia toxicológica. Los cambios histológicos encontrados fueron leves y con frecuencias similares en los tres grupos. Por lo que se concluyó que el adyuvante AFCo1 nasal es potencialmente no tóxico para uso en humanos, por la ausencia de signos locales y sistémicos de toxicidad en las ratas Sprague Dawley.

Palabras clave: *Neisseria meningitidis*, Sprague Dawley, dosis repetida, proteoliposoma.

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