ORIGINAL ARTICLE

Molecular mimicry between major antigens from *Neisseria meningitidis* B and self-proteins as insufficient condition for triggering early post-vaccination autoimmunity

Imitación molecular entre los principales antígenos de la *Neisseria meningitidis* B y las autoproteínas, condición insuficiente para desencadenar autoinmunidad temprana después de la vacunación

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ABSTRACT

Introduction: the concern that certain vaccines may induce autoimmune disease has been conjectured and one of the proposed mechanisms is molecular mimicry (Mm) between vaccine antigens and self-structures. We investigated if Mm for T epitopes of

PorB and other major proteins from *Neisseria meningitidis* B (NMB), contained in a nanoparticle type cochleate
(AIF-nCh), and self-proteins, are able to trigger early autoimmunity reactions in vaccinated C57BL/6 mice.
Methods: Mm between *N. meningitidis* PorB, HmbR and FrpB, and human/mouse proteins was investigated using the bioinformatic tools: SWISS-PROT/TrEMBL SYFPEITHI and FASTA data bases. C57BL/6 mice were immunized intranasally or intramuscularly with AIF-nCh or vehicle using different treatment protocols. Clinical signs were recorded daily and body weight, every 15 days. Mice were sacrificed on day 60, and full necropsy was performed including microscopic studies, leukocyte count, T CD4+ and TCD8+ cell quantification in local lymph nodules and anti-dsDNA antibody levels by ELISA were determined.
Results: Mm was found in several self-proteins from: blood, liver (including fetal liver), skin, brain, lungs, and testicles (human and mouse). Significant alterations of the endpoints evaluated were not detected in any of the vaccinated mouse, except some lymphoid follicles with germinal centers in the draining lymph node, due to lymphocyte activation induced by the normal immune response to the adjuvant formulation.
Conclusions: despite the existence of Mm between PorB and other proteins from NMB, and self-proteins there was no evidence of organic damage or any type of pathological reaction, in mice vaccinated under our experimental conditions.

Key words: vaccine, molecular mimicry, autoimmunity, PorB, HmbR, FrpB, *Neisseria meningitidis* B, bioinformatics.

RESUMEN

Introducción: la preocupación sobre la posibilidad de que ciertas vacunas puedan inducir enfermedades autoinmunes se conjetura y uno de los mecanismos propuestos es la imitación molecular (Mm) entre los antígenos vacunales y las autoestructuras. Se investigó si la Mn para los epitopos T de PorB y otras proteínas fundamentales de la *Neisseria meningitidis* B (NMB) contenidas en una nanopartícula del tipo cocleato (AIF-nCh), y las autoproteínas eran capaces de desencadenar reacciones tempranas de autoinmunidad en ratones vacunados con C57BL/6.
Métodos: se investigó la Mm entre *N. meningitidis* PorB, HmbR y FrpB, y proteínas de humanos y ratones mediante las herramientas bioinformáticas: SWISS-PROT/TrEMBL SYFPEITHI y las bases de datos FASTA. Se inmunizaron por vía intranasal e intramuscular a ratones C57BL/6 con AIF-nCh o vehículo mediante distintos protocolos de tratamiento. Los signos clínicos se registraron a diario y el peso corporal cada 15 días. Los ratones fueron sacrificados a los 60 días. Se realizó la necropsia total que incluyó a los estudios microscópicos, conteo de lecucocitos, cuantificación celular de CD4+ y TCD8+ en los nódulos linfáticos locales y niveles de anticuerpos anti-dsDNA por ELISA.
Resultados: la Mm se detectó en varias autoproteínas del hígado (incluso del hígado fetal), de la piel, pulmones y testículos (de humanos y de ratones). En ninguno de los ratones vacunados se encontraron alteraciones significativas de los puntos terminales, con la excepción de algunos folículos linfoideos con centros germinales en el nódulo linfoide de drenaje, debido a la activación de los linfocitos inducida por la respuesta inmune normal a la formulación adyuvante.
Conclusiones: a pesar de la existencia de Mm entre PorB y otras proteínas del NMB y

de las autoproteínas, no se constató de daño orgánico o ningún tipo de reacción patológica en los ratones vacunados bajo las condiciones experimentales del presente estudio.

Palabras clave: vacuna, simulación molecular, autoinmunidad, PorB, HmbR, FrpB, *Neisseria meningitidis* B, bioinformática.

INTRODUCTION

Vaccines are still the best option to deal with pathogenic agents for the prevention of infectious diseases. Active immunization stimulates the immune system to produce antigen-specific humoral and cellular immunity, but since autoimmune diseases (AIDs) also involve the immune system stimulation against certain antigens of the individual, it is not surprising that some concerns have arisen as to whether immunizations may lead to the development of AIDs.[1,2] Many investigators have extensively explored a possible relationship between immunizations and the occurrence of AIDs, but no conclusive evidence emerged from available data.[3,4] Nowadays, there is no scientific evidence implying that vaccinations can be directly associated with the development of autoimmune diseases, although several isolated reports exist of autoimmune adverse reactions following some vaccinations.[5,6] However, the observed increase in incidence of different autoimmune diseases, the poor understanding of the underlying mechanisms and the growing use of vaccines served as an impetus for more research in this area. It has been suggested that vaccination against pathogenic agents may activate pathways of molecular mimicry (Mm) and immunological cross-reactivity in genetically susceptible hosts and this may be the basis of adverse autoimmune reactions to vaccines.[7-9]On the other hand, Mm between microbial and host structures can be one of the strategies that microbes use to evade aspects of immune killing permitting colonization and invasive disease, and in addition, causing substantial problems for vaccine design.[10,11]

Neisseria meningitidis B (NMB) remains an important cause of severe sepsis and meningitis worldwide. The bacterium is only found in human hosts, and must therefore, continually coexist with the immune system.[10,11] Current vaccines against NMB are based on meningococcal outer membrane (OM) proteins present in outer membrane vesicles (OMV).[12,13] Porins are the most represented OM proteins in pathogenic Neisseria species, functioning as pores for ion exchange.[14] Of these, PorB is the second most prevalent in NMB. It is a pore-forming protein that can translocate to the host-cell membrane during NMB infections.[15,16] It is also an inhibitor of apoptosis in various cell types[17] and a well-established toll-like receptor 2 ligand. PorB is a promising vaccine adjuvant candidate, due to its ability to enhance T-cell co-stimulatory activity of antigen-presenting cells both *in vitro* and *in vivo*.[18] PorB and other porins have also shown immunomodulatory properties acting as potent adjuvants in protecting and safe experimental vaccine candidates in homologous[19] as well as heterologous vaccine scenarios.[20]

HmbR and FrpB are important NMB proteins related with the iron adquisition mechanisms of the bacteria, therefore are essential vaccine antigens candidates.[21,22]

In silico studies (Bioinformatics) might be useful in predicting human toxicity of novel vaccines and in designing non-clinical safety studies.[23] Given that adjuvants typically support the development of new subunit vaccines, bioinformatic analyses can provide first indication of antigen homology (amino acid or DNA sequence of candidate antigen) with self-antigens. Through this evaluation, antigen homology that would theoretically lead to autoimmune response by Mm can be assessed and taken into consideration for selection of the final candidate antigen.[24]

With the aim of studying if Mm itself is able to induce early toxicity, protein PorB from NMB, which is part of the antigen structure and the core of many vaccine formulations, was analyzed using a computer-assisted strategy, for the detection of possible Mm with human and mouse proteins, mapping the more susceptible anatomic sites for developing autoimmune effects (theoretical). In this article, the results of a non-clinical safety study of an experimental vaccine formulation obtained from OMV of NMB are presented. A nanoparticle type cochleate named Adjuvant IFAL-Finlay neo-cochleate (AIF-nCh) that contains PorB in its antigen structure was used.[19]

METHODS

In silico study

To investigate Mm between PorB, HmbR and FrpB of NMB and human or mice protein, a systematic study of sequence similarity was done according to the following algorithm:

Determination of the amino acid sequence

Amino acid sequence of three major antigens (PorB, HmbR and FrpB) were determined with the UniProtKB/Swiss-Prot, data base (http://www.uniprot.org), using the name of the antigen and *Neisseria meningitidis* serogroup B CU385 as descriptors. The entry name selected were: F0AV50_NEIME, with 935597 [NCBI] as taxonomic identifier for PorB, F0ARR9_NEIME, 935597 [NCBI]for HmbR, and Q9JXL3_NEIMB, 491[NCBI], for FrpB. The antigens and others are used in VA-MENGOC-BC, the Cuban antimeningococcal vaccine against serogroups B and C of *N. meningitidis*, and its congener AIF1-nCh (Adyuvant IFAL* - Finlay-neo cochleate).

The sequences used were the following one:

PorB (331 AA)

10	20	30	40	50	60
MKKSLIALTL	AALPVAAMAD	VTLYGTIKAG	VETSRSVEHN	GGQVVSVETG	TGIVDLGSKI
70	80	90	100	110	120
GFKGQEDLGN	GLKAIWQVEQ	KASIAGTDSG	WGNRQSFIGL	KGGFGKLRVG	RLNSVLKDTG
130	140	150	160	170	180
DINPWDSKSD	YLGVNKIAEP	EARLISVRYD	SPEFAGLSGS	VQYALNDNAG	KYNSESYHAG
190	200	210	220	230	240
FNYKNGGFFV	QYGGAYKRHV	RVDENVNIEK	YQIHRLVSGY	DNDALHASVA	VQQQDAKLVE
250	260	270	280	290	300
DNYSHNSQTE	VAATLAYRFG	NVTPRVSYAH	GFKGLFDDAD	LSNDYDQVVV	GAEYDFSKRI
310	320	330			
SALVSAGWLQ	EGKGENKFVS	TAGGVGLRHK	F		

HmbR (786 AA)

10	20	30	40	50	
MLPIAALVGS	IFGNPVLAAD	EAATETTPVK	AEIKAVRVKG	QRNAPAAVER	VNLNRIKQ
70	80	90	100	110	
IRDNKDLVRY	STDVGLSDSG	RHQKGFAVRG	VEGNRVGVSI	DGVNLPDSEE	NSLYARYQ
130	140	150	160	170	
NSSRLSIDPE	LVRNIEIVKG	ADSFNTGSGA	LGGGVNYQTL	QGRDLLLDDR	QFGVMMKN
190	200	210	220	230	
STRNREWTNT	LGFGVSNDRV	DAALLYSQRR	GHETESAGNR	GYAVEGEGSG	ANIRGSAF
250	260	270	280	390	
PDSSKHKYHS	FLGKIAYQIN	DNHRIGASLN	GQQGHNYTVE	ESYNLTASSW	READDVNF
310	320	330	340	350	
NANLFYEWMP	DSNWLSSLKA	DFDYQKTKVA	AVNNKGSFPM	DYSTWTRNYN	QKDLDEIY
370	380	390	400	410	
SMDTRFKRFT	LRLDSHPLQL	GGGRHRLSFK	TFVSRRDFEN	LNRDDYYFSG	RVVRTTSS
430	440	450	460	470	
HPVKTTNYGF	SLSDQIQWND	VFSSRAGIRY	DHTKMTPQEL	NAECHACDKT	PPAANTYF
490	500	510	520	530	
SGFVGLAAQL	NQAWRVGYDI	TSGYRVPNAS	EVYFTYNHGS	GNWLPNPNLK	AERSTTHI
550	560	570	580	590	
LQGRSEKGML	DANLYQSNYR	NFLSEEQKLT	TSGTPGCTEE	NAYYGICSDP	YKEKLDWQ
610	620	630	640	650	
NIDKARIRGI	ELTGRLNVDK	VASFVPEGWK	LFGSLGYAKS	KLSGDNSLLS	TQPLKVIA
670	680	690	700	710	
DYESPSEKWG	VFSRLTYLGA	KKVKDAQYTV	YENKGWGTPL	QKKVKDYPWL	NKSAYVFL
730	740	750	760	770	
GFYKPAKNLT	LRAGVYNLFN	RKYTTWDSLR	GLYSYSTTNA	VDRDGKGLDR	YRAPGRNYAV SLEW

FrpB (714 AA)

10	20	30	40	50	6
MNTPLFRLSL	LSLTLAAGFA	HAAENNAKVV	LDTVTVKGDR	QGSKIRTNIV	TLQQKDESTA
70	80	90	100	110	12
TDMRELLKEE	PSIDFGGGNG	TSQFLTLRGM	GQNSVDIKVD	NAYSDSQILY	HQGRFIVDPA
130	140	150	160	170	18
LVKVVSVQKG	AGSASAGIGA	TNGAIITKTV	DAQDLLKGLD	KNWGVRLNSG	FASNEGVSYG
190	200	210	220	230	24
ASVFGKEGNF	DGLFSYNRNN	EKDYEAGKGF	RNNFNGGKTV	PYSALDKRSY	LAKIGTSFGL
250	260	270	280	290	30
GDHRIVLSHM	KDQHRGIRTV	REEFTVGGDK	ERISMERQAP	AYRETTQSNT	NLAYTGKNLG
310	320	330	340	350	36
FVEKLDANAY	VLEKERYSAD	DSGTGYAGNV	KGPNHTQITT	RGMNFNFDSR	LAEQTLLKYG
370	380	390	400	410	42
INYRHQEIKP	QAFLNSQFKI	EDKEKATDEE	KNKNRENEKI	AKAYRLTNPT	KTDTGAYIEA
430	440	450	460	470	48
IHEIDGFTLT	GGLRYDRFKV	KTHDGKTVSS	NNLNPSFGVI	WQPHEHWSFS	ASHNYASRSE
490	500	510	520	530	54
RLYDALQTHG	KRGIISIADG	TKAERARNTE	IGFNYNDGTF	AANGSYFWQT	IKDALANPQN
550	560	570	580	590	60
RHDSVAVREA	VNAGYIKNHG	YELGASYRTG	GLTAKVGVSH	SKPRFYDTHK	DKLLSANPEE
610	620	630	640	650	66
GAQVGRTWTA	SLAYRFQNPN	LEIGWRGRYV	QKAVGSILVA	GQKDRNGKLE	NVVRKGFGVN
670	680	690	700	710	
DVFANWKPLG	KDTLNVNLSV	NNVFNTFYYP	HSQRWTNTLP	GVGRDVRLGV	NYKF

Prediction of T CD4 and CD8 epitopes SYFPEITHI (version 1.0,1) (http://www.syfpeithi.de) was used to determine human CD8+ T epitopes for HLA-A*0201, and mouse CD8+ T epitopes for H2-Kd. There were selected peptides of 9 amino acids with a score > 20.

Determination of Mm between T CD4 and CD8 epitopes of PorB, HmbR and FrpB *and human/mice proteomes*

Detection of Mm between PorB, HmbR and FrpB and human/mouse proteins, including tissular location of these proteins was performed with the FASTA utility (http://www.uniprot.org).

Adjuvant formulation

Selected antigenic proteins from NMB, including PorB, HmbR and FrpB were extracted and purified according to a standardized method starting from NMB OMV [Outer Membrane Vesicles] supplied by the vaccine production unit of Finlay Institute, Havana, Cuba. Briefly, the proteins were mixed with immunomodulators having defined structures, 1,2-dioleoylphosphatidylserine (DOPS) and cholesterol (Sigma Aldrich Co.) were added to obtain proteoliposomes using a basic methodology,[25] with

little modifications. Nanoparticles were obtained as cochleates adding calcium chloride (0.1 M) to proteoliposomes[26] and were then formulated in suspension using sodium chloride solution (0.9 %) as vehicle and thiomersal as preservative under sterile conditions. The formulation obtained was named AIF1-nCh (Adyuvant IFAL* - Finlay-neo cochleate) and preserved at 4 ºC and protected from light.

Animals and experimental procedures

Specific pathogen-free female and male C57BL6 mice (6-8 weeks old, weighing 20-25 g) were obtained from the National Center for the Production of Laboratory Animals (CENPALAB Spanish acronym), Cuba and housed individually at 23 °C with a 12 h lightdark cycle and one-week adaptation period to animal facility conditions prior to the initiation of the study. The environmental conditions set were: temperature of 23 ± 1 ºC, relative humidity of 55 ± 5 %, and a 12 h light/dark cycle. All experiments were approved by the Institutional Ethical Committee of the Center of Bio-Products, Cuba and performed in accordance with the National Research Council Guidelines for the Care and Use of Laboratory Animals. Mice were randomly assigned to each group (n= 10) according to the experimental design for intranasal (i.n.) or intramuscular (i.m.) administration, and a final intradermal (i.d.) booster, as shown in the table 1.

Poliacrylamide Gel Electrophoresis (SDS-PAGE).

Samples were incubated at 100 ºC for 5 min in presence of 2β-mercapto-ethanol (MercK, Germany) and SDS. Electrophoresis procedure took place using a 12.5 % poliacrylamide gel, followed by a Coomassie R 250 staining. For calculation purposes a Molecular Weight Patterns (MW, Bio-Rad), was applied in parallel with the samples.

Demonstration of specific immune response by Immunotransfer-western blotting

Electrophoretic transference took place in a submerged chamber (BioRad, EUA) during 2 h at 4 ºC, and applying 100 volts. A nitrocellulose membrane (Hybond™-C extra, EUA) was used. Skim milk at 5 % in Phosphate Buffered Saline, 0.15 mol/L, pH 7.2 (SSTF),during 1 h, at room temperature , was used to block free sites in the membrane. After 5 times washing the NC-membrane with Phosphate Buffered Saline, 0.15 mol/L, pH 7.2 (SSTF),it was incubated with the sera from immunized mice at a working dilution of 1:1 000, diluted in SSTF with Skim milk at 1 % and Tween 20 (Sigma, EE. UU.) at 0.01 % (v/v), the incubation time was 14-16 h at 4 ºC, The second antibody used for immunoblotting was goat anti-mice IgG conjugated with peroxidase at 1:1 000 dilution *(*PIERCE*),* development was realized with chemiluminiscent reagent ECL Super Signal® West Dura, (ThermoScientific, EE. UU.), photographic films and a development apparatus.

Clinical observation, body weight and sample preparation for analysis

All animals were observed daily and the incidence of morbidity, mortality, as well as general appearance and detailed clinical signs (behavior such as hyperactivity, lethargy, aggressiveness, piloerection, salivation, diarrhea, nasal discharge, alopecia) recorded. Once a week, injection sites were palpated to detect signs of pain and examine swelling. Body weight of every animal was measured every 15 days throughout the study. At the end of the experiment, before euthanasia and under ether anesthesia, about 1mL blood was collected for haematological studies from the retro-orbital venous plexus of the mice using heparinized capillary tubes.

Hematology

Blood samples were analyzed for white blood cell counts. Lymphocyte and neutrophil counts were determined by extension of a blood drop on a glass slide, then dried, fixed with methanol and stained with Giemsa. The total number of granulocytes and lymphocytes was estimated from the differential count in stained preparations under a binocular microscope.

T lymphocyte immunephenotyping

The sub-iliac lymph nodes for animals immunized via im route, and the cranial deep cervical, superficial parotid and mandibular lymph nodes of those immunized via in route (3 mice from each group) were pooled and macerated in saline phosphate buffer (PBS) + fetal calf serum (1 %) using two glass slides polished at the ends. The cells obtained were passed through a sieve using a cell grinder (mesh 60). After washing the cells twice with PBS, they were counted in a Neubauer chamber and adjusted to optimal concentration (10^6 cells/mL). Anti-mouse CD4-PE-Cy5 (Phycoerythrin-Cychrome5) and anti-mouse CD8-PE (Phycoerythrin), monoclonal antibodies (Pharmingen, San Diego, USA)were used to evaluate the expression of cell surface molecules. The expression of these markers was monitored by flow cytometry using FACScalibur (Becton Dickinson, Mountain View, CA, USA); 30 000 events were acquired. The results were evaluated with the template created in the program Cell Quest Pro for McIntosh (Becton Dickinson) for each cellular sub-population. FlowJo software was used for the analysis of flow cytometry data. The readings were carried out by triplicate.

Assay of anti-dsDNA antibody levels

Anti-DNA autoantibody levels (dsDNA) were determined in sera for all groups by ELISA at the end of experiment,. Micro-ELISA plates (Maxisorp®) from Nalge Nunc International Co. (Naperville, USA) were coated with calf thymus double-stranded (ds) DNA (Sigma) and incubated at 4 ºC overnight. The plates were washed with 0.05 % Tween-20 in PBS. Unbound sites were blocked by incubation at room temperature with PBS containing 1 % milk. Serum samples were diluted 1/4 in PBS containing 1 % milk and 0.05 % Tween-20. The diluted serum samples were added to the plates and incubated at room temperature for 1h, then washed. Anti-mouse goat IgG conjugated with horseradish peroxidase (Sanofi-Pasteur Diagnostics, Marnes La Coquette, France) was added. After 30 min, the plates were washed and tetramethylbenzidine was added. The reaction was then stopped by the addition of 1 M HCl and absorbance at 450 nm was measured with an ELISA reader (Pharmacia).

Necropsy and histopathological examination

A full necropsy was performed on all sacrificed animals. Histopathological examination was performed in samples from the administration site and skeletal muscle (left hind paw) for groups treated im and for groups treated intranasally; the nasal cavity, liver, pancreas, large intestine, salivary glands (parotid and sub-maxillary), bone marrow, thymus, spleen and regional lymph nodes (mesenteric, deep inguinal, popliteal, cervical, parotid and submaxillary), testicles, brain and skin of the control and treated groups, which were fixed in 10 % neutral buffered formalin and processed using routine histological techniques. The testis and epididymis were fixed in Bouin's solution for approximately 24 h and stored in 70 % ethyl alcohol for several days. The nasal

cavities of animals from the corresponding groups were prepared following the method of Gizurarson.[27] After paraffin embedding of the specimens, 3 µm sections were cut and stained with hematoxylin and eosin. The slides were examined microscopically for evidence of cellular damage and inflammation.

Statistical analysis

All dat006)sis uated in a recent study wihn he metabolism cyt P40 mediateda processed are reported as means ± SD. The comparison of the results from various experimental groups and their corresponding controls was carried out using a one-way analysis of variance followed by Bonferroni test. Effects were considered significant if $p \leq 0.05$. Statistical analyses were performed using Statgraphics Plus 5.1 software (StatSoft).

RESULTS

In silico study

Molecular mimicry between T epitopes of PorB, HmbR and FrpB and proteins present in blood, skin, brain, liver (fetal and postnatal) and testicles of human proteome, and in the mouse proteome were detected with the algorithm employed and are shown in table 2.

Clinical observation and body weight

There was no mortality and no relevant clinical signs appeared in any group or time point. There were no treatment-related effects on food intake or water consumption and there were no treatment-related or statistically significant differences in the mean body weights of males or females from any treatment group compared to the control group. A sustained increase in body weight was registered during the study (Fig. 1).

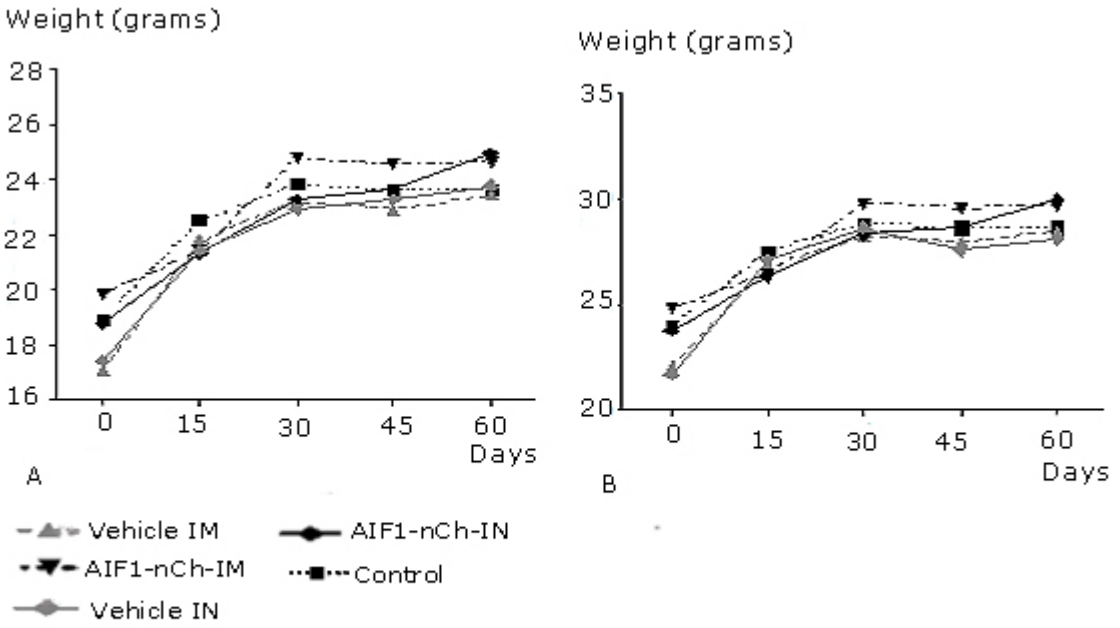

Fig. 1. The body weigth after administration of AIF1-nCh via i.n./i.d. or i.m./i.d. routes in: A) famale, B) male of C57BL6 mice. No statistical differences were found between treated and controls groups.

Hematology and T lymphocyte immunophenotyping

There were no treatment-related or statistically significant differences between the means of total leukocyte, lymphocyte and neutrophil counts of males or females from any treatment group compared to control groups. As a result of the determination of the relative percent of TCD4+ and TCD8+ lymphocytes with regard to the total lymphocytes, we found that in the groups of mice immunized with AIF1-nCh by both im and in routes, the percentage of TCD4+ and TCD8+ lymphocytes was higher than in the control and vehicle groups (table 3).

Anti-dsDNA antibody levels

No statistically significant effect of AIF1-nCh was seen on the levels of anti-dsDNA between the five groups of mice evaluated (Fig. 2).

Necropsy and histopathological examination

There were no macroscopic or microscopic treatment-related effects on the evaluated organs of males and females across all dose groups, including the administration (nasal epithelium or skeletal muscle of the left hind paw) sites, in which inflammatory reactions were not detected. However, cortical lymphoid follicles and well defined germinal centers were observed in the lymph nodes draining the inoculation site (Fig. 3).

Demonstration of specific immune response by Immunotransfer-western blotting

Very strong immuno response against major OMPs: PorA, PorB, HmbR and FrpB, among others was demonstrated as can be seen in Figure 4: Immuno response against other proteins was also shown, but at a lower level and longer exposition times.

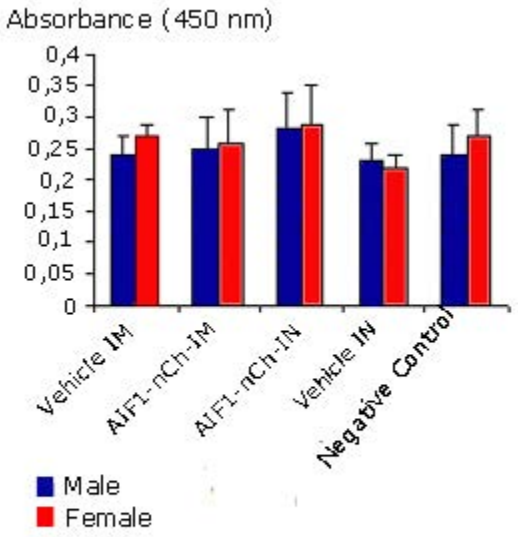

Fig. 2. Effect of AIF1-nCh treatment on anti-dsDNA titer of C57BL6 mice groups. No statistical differences were found between treated and controls groups.

DISCUSSION

The development of autoimmune diseases following vaccination is a matter of debate and one of the mechanisms proposed includes Mm between antigens and "self" structures. According to this hypothesis, when linear and/or conformational sequences are shared by microbial/viral agents and "self" molecules, autoimmunity may occur if the host immune response against the infectious agent cross-reacts with host "self" sequences,[7-9,28] when the host determinants are similar enough to cross-react, yet different enough to break immunological tolerance,[28] in genetically susceptible hosts.

In this investigation, the bioinformatic study revealed several T CD8+ epitopes of NMB PorB sharing sequential mimicry with proteins in different mouse and human tissues. The mimicry observed with fetal liver 1-acyl-sn-glycerol-3-phosphate acyltransferase gamma is irrelevant because during the ontogenetic period, tolerance mechanisms eliminate the majority of auto reactive clones with potential ability to trigger an autoimmune response in postnatal life. On the other hand, mimicry integral membrane protein CII-3b in blood is also unlikely to stimulate an autoimmune effect, due to constant circulation and confrontation in the central organs where immune tolerance is induced and surely, the probability of finding a specific auto reactive lymphocyte is very low. In fact, the rest of the proteins localized in brain, skin, liver and testicles are the focus of our attention.

Besides Mm, a less specific activation of the innate immune response can also promote autoimmune disease in a favorable genetic environment.[29] Adjuvants are used to improve the immunogenicity of vaccine antigens,[30] but this strong unspecific immunostimulating capacity can also cause immunotoxic effects like the induction or worsening of autoimmune reactions.[31] The powerful adjuvant activity of proteoliposome-derived cochleates[32] together with the presence of antigens showing Mm with host molecules are, in theory, two important elements, for the induction of autoimmunity.

Here we studied AIF1-nCh, consisting of cochleate nanoparticles -containing NMB major proteins including PorB, HmbR and FrpB using two different administration routes in mice, to detect early toxicity signs in tissues, where the mimetic proteins are located, after vaccination. The sustained increment of body weight in all treated groups without differences regarding the control groups and the absence of clinical symptoms of toxicity after immunization is a first evidence of animal welfare. On the other hand, the behavior of the hematological end points shows that in the groups treated with AIF1-nCh there was no significant inflammatory response, typical of autoimmune processes.

Adaptive immunity depends on T-cells leaving the thymus and T and B cells travelling between secondary lymphoid organs to survey for antigens. After activation in lymphoid organs, T cells must return to the circulation to reach sites of infection. In the evolution of the immune response during our experiment, some changes were observed, which were interpreted to be associated with the adjuvanticity of AIF1-nCh. The lack of inflammatory cells in the administration sites and the presence of lymphoid follicles with germinal centers in the draining lymph nodes related with injection sites, observed at the end of this study on day 60 reveal that the formulation was rapidly cleared from the administration site and there was no long-lasting local recruitment of immune cells, which have effectively migrated to the draining lymph nodes, as observed by other authors.[33,34]

The reaction observed in local lymph nodes was insufficient to modify general hematological endpoints. Nevertheless, the study of relative numbers of T cells (CD4 and CD8) by flow cytometry, one of the methods recommended in non-clinical safety evaluation of novel vaccines and adjuvants,[23] reveals a stimulation of TCD4 and TCD8 lymphocytes in the groups treated with AIF1-nCh by both administration routes. This result was considered normal, keeping in mind that in these groups of animals the mechanisms of immune response have been stimulated, due to the antigenic stimulus induced by the adjuvant formulation.

The immunogenic property of AIF1-nCh results from the presence of biologically active antigens in their membrane, together with defined immunostimulating molecules mediating either a stimulation of CD4 T cells (MHCII pathway) or CD8 cells (MHCI pathway) mainly in the local lymphoid organs, as almost all adjuvants do.[34] In this study, the nearest lymph nodes to the respective administration site were examined. In all groups immunized with AIF1-nCh, the percentage of TCD4+ cells, was higher than in control non-immunized groups, showing an expansion of these sub-populations for the activation of effective Th1- type immune response, as described for other nanoparticles.[30]

The absence of inflammatory response or any other histological sign of toxicity in those organs and tissues that show Mm with PorB of NMB evidences that there is no early

organ-specific autoimmune response under our experimental conditions. This result agrees with another study performed in our laboratory using a different adjuvant formulation consisting in an OMV cochleate containing PorB named Adjuvant Finlay Cochleate 1 (AFCo1). In this study spermatic toxicity was not observed after repeated intranasal doses in mice.[35]

Although no relationship could be firmly established between serum autoantibody levels and the development or severity of autoimmune diseases, autoantibodies are widely considered the hallmark of autoimmunity.[36] In our study, the titers of anti-dsDNA autoantibodies in serum after AIF1-nCh administration were evaluated, no significant differences among experimental groups were observed, indicating the lack of evidences of autoimmune reactions under our experimental conditions and regarding this indicator.

Considering all the results together, there was no evidence of autoimmune signs as consequence of mimicry in the model used. Although, it is important to consider that major difficulties when addressing autoimmunity include that the mechanisms involved are not fully understood, and no validated models are available.[24,36] Several months to years can elapse between priming of the adaptive immune system against a host tissue and the relevant organ destruction resulting in manifest autoimmune disease, in a multistep process.[37] While no single factor can be identified as the leading cause of autoimmunity, interplay of factors involving specific MHC patterns and environmental factors, besides Mm, seem to be the most accepted hypothesis.[38-40] Therefore, the absence of immunotoxicity signs in this study does not absolutely rule out the possibility of induction of a late post-immunization autoimmune phenomenon.

An alternative analysis can be addressed with our results. Breaking powerful self-tolerance mechanisms that avoid harmful self-reactivity seems unlikely when high degrees of similarity are present between non-self and self molecules. Thus, sharing epitopes with host molecules may represent an elective mechanism used by NMB to escape immune attack.[41] The most striking mimicry, occurs in serogroup B capsule, in which the α(2-8)-linked sialic acid homopolymer is structurally identical with a component of human NCAM (neural cell-adhesion molecule), crucial for functional plasticity of the central and peripheral nervous systems.[10,11] Such identity is responsible for the particularly poor immune response generated against serogroup B capsule by humans.[11]

The Cuban VA-MENGOC-BC® vaccine, is an Outer Membrane Protein-based serogroup B meningococcal vaccine, which has been the most successful formulation against NMB with an excellent record of protection and safety. This vaccine contains several NMB antigenic proteins including PorB, HmbR and FrpB,among other Outer Membrane Proteins. More than 70 million doses have been administered in Cuba and other countries to millions of persons of different age groups and even after more than 25 years of follow up there are no reports of autoimmune post-vaccination reactions.[12,42] These results in humans are mutually reinforced with the negative findings for this new formulation first tested in mice.

Very strong immuno response against major OMPs: PorA, PorB, HmbR and FrpB, among others were demonstrated by Immunotransfer-western blotting. Also, the immuno-protective character of this response have been extensively proved (Results not shown here). This strong specific immune response is the desired face of the

results, it constitutes the main characteristic of a good vaccine candidate; In spite of that, no adverse reactions or evidences of autoimmunity have been observed.

In conclusion, our study supports that in spite of the existence of Mm between the PorB HmbR and FrpB, of NMB and self proteins, the probability for triggering an early autoimmune disease post-vaccination seems very limited. Nevertheless, future studies using genetically prone autoimmune models and long lasting observation can offer valuable additional information under special conditions.

* Product and method patent requested.

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Table 1. Experimental design for a toxicity study of AIF1-nCh in C57BL6 mice

Groups	Doses/Volume	Route of administration	Day of administration	Female	Male
Vehicle (SSF + Thimerosal 0.005 %)	250 µL (2 doses)	i.m.	0-14	10	10
	30 µL (1 dose)	i.d.	21		
AIF1-nCh-IM	15 µg/250 µL (2 doses)	i.m.	0-14	10	10
	15 µg/30 µL (1 dose)	i.d.	21		
AIF1-nCh-IN	15 µg/25 µL/nostril (3 doses)	i.n.	0-7-14	10	10
	15 µg/30 µL (1 dose)	i.d.	21		
Vehicle (SSF + Thimerosal 0.005 %)	25 µL/nostril (3 doses)	i.n.	0-7-14	10	10
	30 µL (1 dose)	i.d.	21		
Negative control*	-	-	-	10	10

*Negative control group was not treated.
Euthanasia was carried out on day 60 of the experiment.

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Table 2. Tissular location of A) human proteins and B) mice proteins with molecular mimicry for PorB, HmbR and FrpB from NMB

A) Antigens	Nonamers	Mimetic human protein	Tissular localization
PorB	D L G N G L K A I	Integral membrane protein CII-3b	Blood
	V A A M A D V T L	1-acyl-sn-glycerol-3-phosphate acyltransferase gamma	Skin, brain, fetal liver, testicle
	G I V D L G S K I	LFIRE 1	liver
	L R V G R L N S V	PRO0907	liver, testicles
HmbR	T L R A G V Y N L	Peroxisomal trans-2-enoyl CoA reductase	liver, fetal liver, placenta
	Q L G G G R H R L	FBXL18 protein	lungs, testicles
	L L D D R Q F G V	Neuronal membrane glycoprotein M6-a	Brain
	S L G Y A K S K L	Asparaginase-like protein	testicles
FrpB	K I R T N I V T L	Ras-related protein Rab-25	Bowel, ovary, skin, testicles
		Treacle protein	Eyes, skin, epitelium
	S L L S L I L A A	Transmembrane protein 179	Brain
	H A A E N N A K V	protein C2orf31	testicles
	I L Y H Q G R F I	NUAK family SNF1-like kinase 2	Testicles, lymphatic system
	A I I T K T V D A	Coiled-coil domain-containing protein 79	testicles
B) Antigens	Nonamers	Mimetic mouse proteins	
PorB	S Y A H G F K G L	protein C7orf31 homolog (Protein TISP74)	
HmbR	K Y H S F L G K I	PIH1 domain-containing protein 1 (Nucleolar protein 17 homolog)	
	N F N S S R L S I	1700012L04Rik protein (Novel protein similar to Histone H2A) Predicted (Novel protein similar to Histone H2A). Adult male testis cDNA, RIKEN fu	
	D Y Y F S G R V V	Calicin	
	H R L S F K T F V	Zinc finger protein 313	
FrpB	A Y I E A I H E I	Adult male testis cDNA, RIKEN fu	

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Table 3. Total leukocytes (x 10^9/L), and relative percentage of lymphocyte, neutrophil and TCD4+/TCD8+ lymphocytes after administration of AIF1-nCh i.n./i.d. or i.m/i.d routes in C57BL6 mice

Groups	Total leukocyte count[a] (x 10^9/L)		Lymphocytes[b] (%)		Neutrophils[c] (%)		Relative percentage of TCD4+/TCD8+ lymphocytes[d]	
	Female	Male	Female	Male	Female	Male	Female	Male
Vehicle	10.2 ± 0.8	10.0± 1.9	90.2 ± 2.9	91.7±2.1	5.5 ±2.5	8.3 ± 3.7	69.2/6.8	70.4/7.21
AIF1-nCh-IM	9.7 ± 0.2	9.7 ± 1.0	89.6 ± 0.5	91.4 ± 0.5	4.6 ± 0.4	8.0 ± 0.1	87.2/10.1	88.6/9.7
AIF1-nCh-IN	10.1 ± 0.7	9.5 ± 0.3	89 ± 0.7	92.2 ± 1.6	5.1 ± 0.6	8.1 ± 0.2	94.9/8.9	95.2/9.1
Control	9.7 ± 0.2	9.1 ± 1.4	89.4 ± 1.1	91.6 ± 1.6	5.2 ± 0.3	8.0 ± 0.2	71.9/7.65	70.8/6.91

[a, b, c] Means and SD of the two groups inoculated with the vehicle by i.m. and i.n. route.
[d] The quantification of $TCD4^+$ and $TCD8^+$ lymphocytes is expressed in terms of percentage of $TCD4^+$ or $TCD8^+$ lymphocytes of the total cells counted in the lymph nodes of the mice from the different experimental groups.
No statistical differences were found between treated and control groups.

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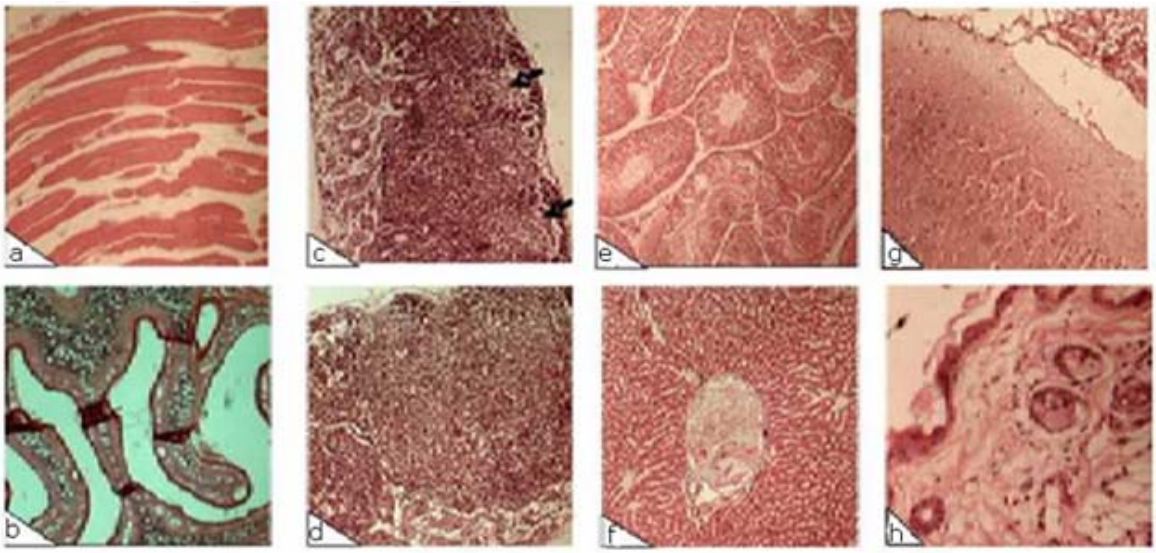

Fig. 3. Selected pictures of histopathological findings after administration of AIF1-nCh i.n./i.d. or i.m./i.d. routes in C57BL6 mice. Inoculation site of a mouse treated with AIF1-nCh without inflammatory response: a) muscle of the left hind paw (i.m. inolucation), b) nasal cavity (i.n. administration) Lymph nodes (i.m. inoculation), c) draining popliteal lymph node d) contralateral popliteal lymph-node (righ hind paw). Non lymphoid organs with molecular mimicry, e) testis, f) liver, g) brain, h) skin of AIF1-nCh-IM showing normal aspect. Similar results were obtained for all the groups.The sections were stained with hematoxylin & eosin and shown under100 x magnifications.

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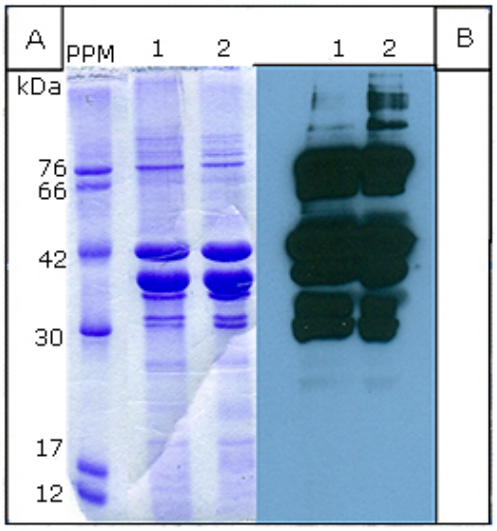

Fig. 4. A: Poliacrylamide gel Electrophoresis with detergent SDS (12.5 % gel). Coomassie Brilliant Blue staining PPM: Molecular Weight Patterns expressed in kDa. Lanes 1 and 2: Purified major Outer Membrane Proteins from *N. meningitidis* B and same proteins re-extracted from the nano-particles of AIF1-nCh, respectively. B: Western-Blotting. Proteins from 12.5 % Poliacrylamid gel transferred to nitrocellulose membrane incubated with sera from AIF1-nCh immunized mice, second antibody used for immunoblotting was goat anti-mice IgC conjugated with peroxidase 1:1 000 (PIERCE), development with chemiluminiscent reagent ECL Super Signal® West Dura, (ThermoScientific, EE. UU.). Lanes 1 and 2 correspond to lanes 1 and 2 of the section A. Very strong immuno response against major OMPs: PorA, PorB, HmbR and FrpB was demonstrated. Immuno response against other proteins was also shown, but at a lower level and longer exposition times.

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