

AFCo1 Induces Mucosal Cross Response Against Polysaccharides Of *Neisseria Meningitidis* Serogroups In Mice



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ABSTRACT

Neisseria meningitidis is a causal agent of human meningitis. Twelve serogroups exist base in their polysaccharide compositions, six of them cause meningococcal disease, plane and covalently conjugated parenteral vaccines are available. The serum IgG cross reactivity between pathogenic and non-pathogenic strains is a fact. It is consequence of their high protein homology. Nevertheless, the mucosal IgA cross response against polysaccharides are unknown. So, the response induce by polysaccharide C from *N. meningitidis* alone of adjuvantated with AFCo1 adjuvant in Balb/c mice was addressed. Three intranasal doses did not induce serum IgG responses against A, Y nor W135N. meningitidis serogroups. Contrary, polysaccharide C alone and better when it is formulated with AFCo1 induce mucosal IgA cross reactive response against all polysaccharide tested. The possible use of SinTimVaS approaches for vaccine development is suggested

Abbreviations: OD: Optical Density, CTL: Cytotoxic T Lymphocyte, BSA: Bovine Seroalbumin

Short Communication

Neisseria is a mucosal obligate pathogen or non-pathogen species where *N. meningitidis* has 13 serogroups. This classification is based in its polysaccharide expression and they are: A, B, C, D, E, H, I, K, L, W135, X, Y y Z. Of them, the A, B, C, W135 and Y serogroups are the most disease causing agents. As polysaccharides are thymus-independent antigens the current approach for vaccines purpose is the covalent conjugation to some proteins and theirs use as parenteral vaccines [1]. Nevertheless, in not immunodeficient human the occurrence of a disease by *N. meningitidis* infection is only one time and it arrives by mucosal route. In addition, the mucosal IgA response induce by *N. meningitidis* infections have been less explored. The IgA response could be induced as a thymus-dependent and -independent manner [2]. The former is mainly induced against proteins antigens and it is more specific. The latter is not totally clear which antigen is the main responsible for its induction and might be more cross reactive. Consequently, we would like to explore the possible cross reactivity induce by polysaccharides applied by mucosal route in mice.

Although *N. meningitidis* infects through mucosal surfaces where IgA is the main protecting antibody, systemic vaccination is currently used which did not induce mucosal response. At present antigen delivery to mucosal surfaces is very interesting especially for protection against infectious agent that are mediated by local immune response. However, mucosal immunization has been limited by important aspects as safety and immunogenicity. An approach to improve this route of vaccination is the use of safe and effective adjuvants. Cross reactivity at serum IgG level between species of *Neisseria* genus, in particular with *N. gonorrhoeae* have been described [3]. However, its main explanation is by their structural homology between commons proteins in those species. One of the shortcomings of parenteral immunization is its relatively poor ability to induce specific IgA [4,5]. Nevertheless, cross reactivity at mucosal IgA level against polysaccharides between *N. meningitidis* serogroups have been less explored [6]. The first vaccine against *N. meningitidis* serogroups B and C is base in the outer membrane detergent extractions of a Proteoliposome (PL)

form the B serogroup [7] which contains several proteins [8,9]. It is bivalent as it contains also the C polysaccharide adsorbed onto Al(OH)₃ (Alum). Our group have been demonstrated that PL in addition to the protective antigens function as a potent adjuvant [10,11], and using this polysaccharide covalent conjugation, the hallmark of polysaccharide vaccines, is not required [12-14]. This vaccine is use by parenteral route overcoming the polysaccharide C thymus-independence also in human toddlers [15]. Nevertheless, as PL is absorbed onto alum it could not be use as mucosal adjuvant. So, we have developed a PL-derived cochleate (AFCo1) adjuvant. AFCo1 is a multilayer microparticle formed by the interaction of the lipid bilayer of the PL and Ca²⁺, which contains similar proteins and pathogen-associated molecular pattern as the PL [10,11] inducing systemic specific IgG and in addition mucosal specific IgA. AFCo1 applied by nasal route induces mucosal at regional and distal places (specific IgA) and systemic immune responses (serum specific IgG, specific IgG2a subclass, and IFN- γ) polarizing to a Th1 pattern [16]. The mucosal approach has being applied to several antigens [17,18].

Materials and Methods

Animals

Female Balb/c mice, six to eight weeks old, supplied by Cenpalab, Cuba were used for all the experiments. All the experiments were performed with the approval from the Ethical Committee for Laboratory at Finlay Institute, Cuba. The studies were performed at least twice.

Preparation of Proteoliposome (PL) and AFCo1

PLs from outer membrane vesicles detergent-extracted from *N. meningitidis* serogroup B were prepared and supplied as an ethanol precipitate, as well as the C polysaccharide, by the vaccine production unit of the Finlay Institute, Havana, Cuba, for *N. meningitidis* serogroup B vaccine as previously described. PL-derived AFCo1 was prepared as previously described [11].

Animal Immunization and Sample Collection

Mice were immunized intranasal (i.n) with 3 doses of AFCo1 + C polysaccharide; polysaccharide alone or naive as negative control with 7 days intervals. In all groups the blood was obtained from the retroorbital vein at 21 days after the last dose. Saliva samples were collected at 7 days after the last immunization by Pilocarpine 0.5% intraperitoneal stimulation. These were immediately inactivated at 56°C for 15 min and the supernatants after centrifugation (9000 g) were stored as well the sera at -20°C.

Analysis of Mouse Antibody Response by ELISA

Maxisorp 96-well plates (Nunc) were coated with 100 μ L of *Neisseria meningitidis* polysaccharide A, C, Y or W135 (10 μ g/mL) in 0.05M carbonate buffer, pH 9.6 for 4 h at room temperature, followed by overnight incubation at 40C. The plates were blocked with 2% Bovine Seroalbumin (BSA) in Phosphate Buffer Saline (PBS) for 30 min at 37°C. The serial dilutions of sera or vaginal

washes obtained were incubated for 1 h at 37°C. After washing with 0.05% Tween-20, plates were incubated 1h at 37°C with goat anti-mouse immunoglobulin (Ig) G, (Southern Biotechnology Associates, Inc., Birmingham, AL), or -mouse IgA (Southern Biotechnology Associates, Inc., Birmingham, AL) coupled to horseradish peroxidase in 1% BSA-PBS buffer. The plates were then washed with 0.05% Tween-20 and developed using 100 μ L of 1 mg/mL o-phenylenediamine dihydrochloride (Sigma) in 0.1 M citrate buffer (pH 4.5) in the presence of 0.04% H₂O₂. The reaction was stopped with H₂SO₄. The absorbance was read at 492 nm and was expressed as Optical Density (OD).

Statistical Analysis

Statistical analyses were done by two way ANOVA followed by Bonferroni post test using Graph Pad Prism 5 software (CA, USA).

Results and Discussion

Neither C Polysaccharide Alone nor Adjuvantated With AFCo1 by Nasal Route Induce Cross Reactive Serum Igg Against Heterologous *Neisseria Meningitidis* Polysaccharides

Low serum anti Polysaccharide C IgG was obtained immunizing mice with it alone. This response was significantly increase by the adjuvantation with AFCo1. No serum specific IgG responses were obtained against heterologous polysaccharides with both formulation (Figure 1). These results are in agreement with the strong adjuvant capacity of AFCo1 against proteins [18,19], and non-covalently conjugated polysaccharides [19,20]. Our previous results have been confirmed that VA-MENGOC-BC™ vaccines as well as its derivatives AFPL1 and AFCo1 induce a preferential Th (helper) 1 pattern of immune response characterized by: induction of IgG and IgG1 (in human, IgG2a in mice) with opsonophagocyte and some bactericidal activities; delayed-type hypersensitivity; and IL-12 and IFN- γ , but neither Th2 (IL-4, IL-5) cytokine production nor mucosal specific IgA response. They also change the Th2 pattern normally induced by AL(OH)₃ to a preferential Th1 immune response in mice and human. Beside, cross-priming and Cytotoxic T Lymphocyte (CTL) responses were induced in mice. Nevertheless, the transformation in AFCo1 meanwhile conserving its component increases the immunogenicity of the antigens of the formulations [10,21,22]. Overall, only the AFCo1 containing formulation increase the systemic IgG anti polysaccharide C response by nasal immunization.

The C Polysaccharide Alone Immunized by Nasal Route Induces Cross Reactive Saliva Iga Against Heterologous Polysaccharides Which is Increased by AFCo1 Adjuvantation

To explore the mucosal IgA response induced by polysaccharide C containing formulations and their possible cross response over heterologous *N. meningitidis* polysaccharides mice were immunized mice with 3 i.n doses of polysaccharide C or adjuvantated with

AFCo1. The polysaccharide C alone induces IgA responses that recognized the homologous and heterologous polysaccharides (Figure 2). These response are only at mucosal IgA level because it neither induce systemic specific nor heterologous cross reactive IgG (Figure 1). The adjuvantation with AFCo1 significant increases the specific homologous and the cross reactive heterologous IgA

anti polysaccharide responses (Figure 2). These results confirm our previous results that a potent adjuvant, like AFCo1, overcome the thymus-independence of polysaccharide C without the requirement of covalent conjugation^{12,13,14}. In addition, AFCo1 also significantly increases the cross reactive IgA induction against heterologous polysaccharides (Figure 2).

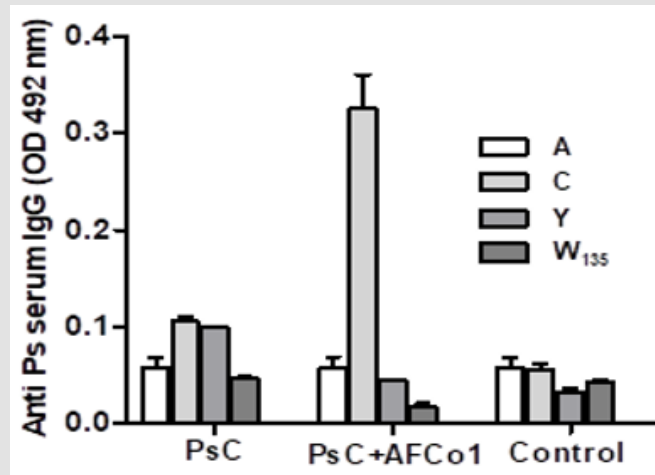


Figure 1: Neither C polysaccharide alone nor adjuvanted with AFCo1 by nasal route induce cross reactive serum IgG against heterologous *Neisseria meningitidis* polysaccharides. A, C, Y and W₁₃₅ are *N. meningitidis* serogroups; PsC *N. meningitidis* polysaccharide used in immunizations, OD, optical density determined by ELISA.

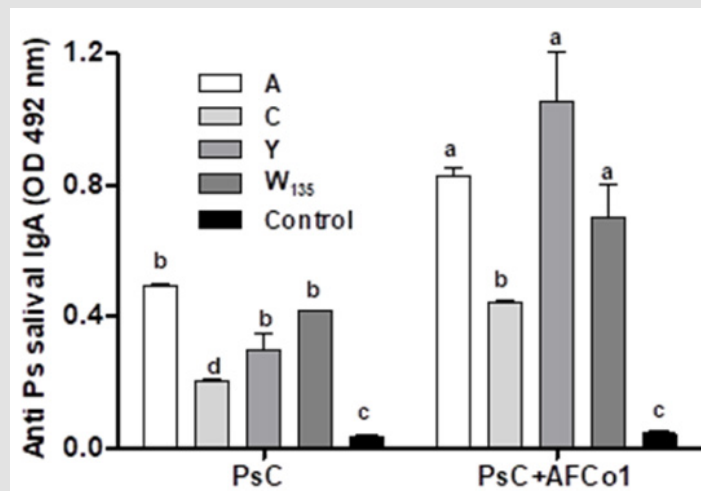


Figure 2: The C polysaccharide alone immunized by nasal route induces cross reactive saliva IgA against heterologous polysaccharides which is increased by AFCo1 adjuvantation. A, C, Y, and W₁₃₅ are *N. meningitidis* serogroups; PsC, *N. meningitidis* polysaccharide used in immunizations, OD, optical density determined by ELISA. Different letters mean significant different value determined by Two way ANOVA followed by Bonferroni post-test.

This IgA cross reactivity could not be explained by polysaccharide structural homology as occurs with the systemic anti proteins IgG response between different *Neisseria* species and serogroups². So, these results open a new window of mucosal vaccination against *Neisseria* serogroups. Nevertheless, as it only induces IgA, but not IgG cross reactive responses the mucosal vaccination approach is not enough to protect against the different meningococcal serogroups that require bactericidal and opsonophagocytic serum antibodies

to protection. So, one alternative could be the use of single time vaccination strategy (SinTimVaS) that combine at the same time two priming, one parenteral and another mucosal. SinTimVaS induces specific mucosal IgA and systemic IgG responses [21-25]. We are exploring this strategy for the induction of both mucosal and systemic cross reactive responses against different *Neisseria meningitidis* serogroups.

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