Biologicals xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Individual variability in humoral response of immunized outbred mice and cross-reactivity with prevalent Brazilian *Neisseria meningitidis* strains

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1. Introduction

Immune response to vaccines may be influenced by many parameters including genetic variation, which seems to have a strong influence in the variability of this response [1]. Immunogenetic studies demonstrated that single nucleotide polymorphisms in human leucocyte antigen (HLA), cytokines and its receptors, innate immune response and cell surface receptor genes can be the involved in the inter-individual variability of immune responses to vaccines [1,2].

Murine models are widely used in biological research; these models supply experimental conditions comparable to results in human. Mice preserve almost 99% of human genes and have a similar physiology [3]. Many immunological researches use inbred mice as models; however, these may not represent the complexity of human population immune responses to vaccines. Outbred mice are available to perform immunological studies and present genetic diversity, similar to that observed in human population [4].

Neisseria meningitidis is one of the main etiological agents of bacterial meningitis, which is a major public health problem worldwide due to its high mortality and morbidity rate [5]. These bacteria are classified into serogroups according to the immunochemistry of its capsular polysaccharides. The meningococcal invasive disease is mainly related to serogroups A, B, C, W, X and Y [6].

The meningococcal disease imposes high costs of treatment and rehabilitation; therefore the prevention by vaccination is cost-effective [7]. The initial diagnosis is impaired by the lack of specific symptoms and many individuals develop severe sequelae [8], making prevention the best strategy to dealing with meningococcal disease.

In countries with established vaccination programs against serogroup C *N. meningitidis* show an increase in the rates of invasive meningococcal disease caused by serogroup B, such as in Australia, Europe and North America [9]. There is a need to develop non-capsular vaccines against *N. meningitidis* from serogroup B due the similarity of the capsular polysaccharides with human polysialylated glycoproteins [10] that may lead to autoimmunity development and low immunogenicity [6]. The vaccines based on outer membrane vesicles (OMVs) are described as highly immunogenic [11]. The OMVs vaccines were used to successfully contain outbreaks in Cuba, Chile, Norway, Brazil and New Zealand [10].

Many diseases are caused by antigenic diverse pathogens, including meningococcal disease, it is important that vaccines for these multi-strain diseases induce cross-reactivity. An effective vaccine should not only reduce the risk of infection with vaccine strains, but also decrease the risk of infection by other strains by cross-protective immunity [12].

This study aimed to evaluate the individual humoral immune response of male and female mice after immunization with *N. meningitis* strain B:4:P1.9 OMVs, and to assess the cross-reactivity of antibodies with Brazilian prevalent strains. We chose Swiss outbred mice as model given the large individual genetic variation among the animals [13].

2. Materials and methods

2.1. Bacterial strain and obtainment of the outer membrane vesicles (OMVs)

We selected the strain B:4:P1.9 to be used in this study. This is a Brazilian epidemic strain from 1988, isolated from a patient who developed systemic infection [14].

Abbreviations: AI, avidity index; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FbpA, ferric binding protein A; FetA, ferric enterobactin A; fHbp, factor H binding protein; HLA, human leucocyte antigen; LPS, lipopolyssacharide; MenB, *Neisseria meningitidis* serogroup B; MW, molecular weight; NadA, Neisseria adhesin A; NHBA, Neisserial Heparin Binding Antigen; ns, non-significant; nt, nontypeable; NspA, neisserial surface protein A; OMPs, outer membrane proteins; OMVs, outer membrane vesicles; Opa, opacity protein; PBS, phosphate-buffered saline; PorA, porin A; PorB, porin B; SEM, standard error of mean; Tbp, transferrin binding protein

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https://doi.org/10.1016/j.biologicals.2018.08.001
Received 17 January 2018; Received in revised form 11 July 2018; Accepted 2 August 2018

The extraction of OMVs was performed as previously described [15]. OMVs were extracted in a buffer containing sodium acetate 0.1 M and lithium chlorite 0.2 M (pH 5.8). The preparation was incubated over agitation for 2 h at 45 °C and then centrifuged at 12.000 × g for 20 min at 4 °C, with glass beads to stimulate blebs release. The supernatant corresponding to OMVs was dialyzed overnight in NaCl 0.15 M at 4 °C. After that, the extraction OMVs were passed through a Sepharose 4B column linked to polymyxin B, to reduce the content of lipopolysaccharide (LPS). Protein concentration was measured in NanoDrop 2000 equipment (Thermo Scientific) and the concentration obtained was 1345 µg/ml.

2.2. Antigenic preparation

To produce the antigenic preparation, we used an aluminum hydroxide suspension (Rehydragel HPA, Reheis) 0.1 mM complexed with10 µg/ml of OMVs diluted in NaCl 1 mM.

2.3. Mice and immunizations

Swiss mice were obtained from Adolfo Lutz Institute. The animals were followed and analyzed individually. Different patterns of holes in the ear were used to identify each animal. When the mice were 21 weeks of life they were immunized with 200 µl of the antigenic preparation via intramuscular injection, into their anterior tibialis muscles, at day 1, 20 and 30. Blood samples were collected from animals by puncture of retro-orbital plexus at day 1, 20, 30 and 45 (Fig. 1). In this study we used three females and five males. Female were identified as 1 to 3 and male as 4 to 8 in the figures. All procedures involving animals were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the ethics committee of CEUA IAL/Pasteur (protocol number 06/2012).

2.4. Immunoblot

The proteins present in the OMVs were separated by electrophoresis in a 13% polyacrylamide gel, in discontinuous system [16], using a concentration of 67 µg of OMVs. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (BIO-RAD Laboratories) at 100 V for 18 h at 4 °C [17]. The membrane was then blocked with skimmed milk (La Sereníssima) 5% in PBS for 2 h. The nitrocellulose membrane was then washed and incubated with the serum samples diluted 1:100, overnight at 4 °C. After washing five times, the membrane was incubated with a phosphatase-conjugated anti-mouse IgG whole-molecule (Sigma-Aldrich) diluted 1:2000 for 2 h over agitation. Then it was washed again and incubated with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma-Aldrich) for 20 min. The reaction was stopped by washing the membrane with water [18].

2.5. ELISA

OMVs from strain B:4:P1.9 were used as coating antigen. The assay was executed as previously described [19]. Briefly, OMVs were resuspended in coating buffer (0.5 M carbonate-bicarbonate, pH 9.6) at concentration of 5 µg/mL, added in each well of microtiter plates (High Binding®, COSTAR™) and left overnight at 4 °C. The plates were washed with PBS 0.1% Tween 20 and blocked with skimmed milk (La Sereníssima) 5% in PBS 0.1% Tween 20 for 2 h at 37 °C. The plates were washed again and the serum samples were added at two-fold dilutions starting with 1:500 and incubated overnight at 4 °C. The plates were washed again and incubated with a peroxidase-conjugated anti-mouse IgG whole-molecule (Sigma-Aldrich) diluted 1:2000 for 2 h at 37 °C. After a final washing, 3,3′,5,5′-Tetramethylbenzidine (TMB) was added for color development, and incubated for 20 min at 37 °C. The reaction was stopped with 1 N H_2SO_4, and the readings were performed at 450 nm using a microplate spectrophotometer (Labsystem Multiskan). This assay also used biotin-conjugated anti-mouse IgG1 (Zymed), peroxidase-conjugated anti-mouse IgG2a (Zymed) and peroxidase-conjugated anti-mouse IgM µ-chain specific (Sigma-Aldrich). The IgG1 analyses included an additional step, after the incubation with the conjugate the plates were incubated with streptavidin-peroxidase (Zymed 1:2000) for 1 h.

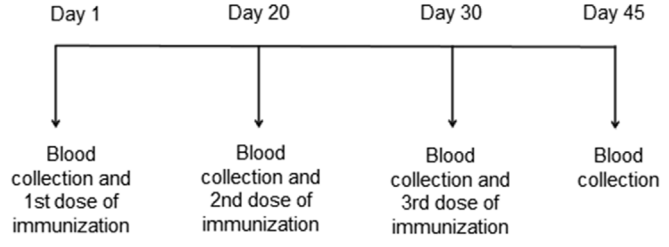

Fig. 1. Immunization scheme and bleeding of outbred Swiss mice.

2.6. Antibody avidity assay

Antibody avidity assay was measured by an ELISA elution using the chaotrope agent potassium thiocyanate (KSCN 1.5 M), as previously described [20]. The procedure was similar to ELISA, however before the addition of the conjugated antibody, we added KSCN to the plates and incubated for 20 min at room temperature. The avidity index (AI) was defined as the average ratio of absorbances in the presence and absence of KSCN multiplied by 100 [21]. Al values greater than 50% indicated high avidity, between 30% and 50% intermediate avidity and below 30% low avidity [22].

2.7. Dot-blot ELISA

Dot-blot ELISA assay was performed to evaluate cross-reactivity of IgG antibodies, produced after immunization, against *N. meningitidis* strains of serogroups B, C, W and Y, isolated from patients of diverse regions of Brazil between 2014 and 2016 (Table 1). The method was performed as previously described [23] with some modifications. We briefly spotted meningococcal cell suspension on a 0.45 µm nitrocellulose membrane (BIO-RAD Laboratories) and left to dry at room temperature. Then the membrane was blocked with skimmed milk (La Sereníssima) 5% in PBS for 2 h. The membrane was washed and incubated with serum samples diluted 1:400 overnight at 4 °C. After washing again, the membrane was incubated for 2 h over agitation; with a phosphatase-conjugated anti-mouse IgG whole-molecule (Sigma-Aldrich) diluted 1:2000. Then, it was washed again and incubated with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma-Aldrich), for 20 min. The reaction was stopped by washing the membrane with water. The results were read visually. Fig. 2 presents the arrangement of strains in the nitrocellulose membrane.

2.8. Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 5 (Graph Pad Software, Inc., La Jolla, CA, USA). The analyses were performed using One-Way ANOVA followed by Tukey test. P values < 0.05 were considered to be significant.

3. Results

3.1. Evaluation of specific reactivity by immunoblot technique

We evaluated the serums from mice after the third dose of immunization individually, to check the differences in the antigenic recognition of the homologous strain. A pool of pre-immune serums was