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Evaluation of Bactericidal Activity of Monoclonal Antibodies Obtained from *Neisseria meningitidis*

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Abstract

Introduction: Serum Bactericidal Assays (SBAs) are considered as the gold standard to evaluate the immunogenicity of many vaccine formulations against infectious agents, for example *Neisseria meningitidis* vaccines. SBAs are also used to evaluate vaccine lots before release to the market, because it has been demonstrated that there is a correlation between bactericidal antibody titers and protection. For Laboratory and Clinical Good Practice, it is very important to have a positive control in each assay. To our knowledge, there is no commercial positive control to serve this function, therefore the purpose of this work was to evaluate a monoclonal antibody (MAb) panel against *N. meningitidis* strains produced at Institute Finlay of Vaccines as a reference material in the established bactericidal assay, with the advantage of high homogeneity and specificity and relative low cost of the MAbs test agents.

Materials and Methods: Specificity of a panel of MAbs was evaluated by a whole cell enzyme linked immunoassay (ELISA). The positive MAbs were then tested for bactericidal effect against target strains: F8238 (serogroup A), CU385/83 y NZ228/98 (serogroup B) and C11 (serogroup C). Determinations were carried out in triplicate and the mean was calculated.

Results: In this study , we positively identified five MAbs out of seven that recognised specific, selected *N. meningitidis* strains. However, only three MAbs (anti-PsA, anti-P1.15 and anti-P1.4) showed bactericidal activity with their homologous strain, and this was related to the MAbs subclass.

Conclusions: Three monoclonal antibodies presented bactericidal activity and they have the potential to be used as positive controls in bactericidal assays.

Keywords: Bactericidal activity; Monoclonal antibodies; *Neisseria meningitidis;* Serum bactericidal assays

Introduction

N. meningitidis is the causative agent of meningoencephalitis and septicemia in adults and children. This gram-negative bacterium contains an outer membrane with several proteins surrounded by a polysaccharide capsule, which constitute the main surface antigens. This microorganism is classified in 12 serogroups, related to the structure of the capsular polysaccharide. Serogroups A, B, C, X, W and Y, are responsible for 90% of the illness cases reported [1,2].

ELISA is simple, economical and highly sensitive, and it is widely used in the diagnosis of infection, evaluation of the MAbs antigenicity and immunogenicity of vaccine candidates in antibody response terms [3-5]. For some vaccine, this method is enough to release vaccine lots, and has been used in replace of other *in vitro* functional test or potency assay [6]. Unfortunately, for meningococcal vaccines the antibody response from ELISA is not enough to identify the vaccine protection or to release vaccine lots to the market, because there is no correlation between human antibody response determined by ELISA and protection [7].

In 1918, the first studies on bactericidal antibodies and their critical role in protection against meningococcal disease were described. Later, in 1969, with the development of the first monovalent vaccine against serogroup C, the correlation between the bactericidal activity and protection was demonstrated [4]. In 1976, a World Health Organization (WHO) Expert Committee recommended the use of a Serum Bactericidal Assay (SBA) to satisfy the requirements for production and release of meningococcal polysaccharide vaccine [8,4].

In 1987, the Institute Finlay of Vaccines (IFV) developed a pioneering vaccine against serogroup B based on an outer membrane protein vesicles strategy, VA MENGOC-BC®. The Institute also developed plain polysaccharide vaccines against serogroups A, C and W. In general, the SBA is used to evaluate the immunogenicity of current meningococcal vaccines and new candidates, because it is considered a Gold standard [9,1]. Given the importance of SBAs, this is also used in the quality control of vaccines for lot release.

Positive controls are very important for Laboratory and Clinical Good Practice [1] and to our knowledge; there is no-commercial positive control for *N. meningitidis* SBA. For this reason, the objective of this study was to evaluate a monoclonal antibody panel produced at IFV as a reference material and positive control in the bactericidal assay, with the advantage of having a high homogeneity, specificity and relative low cost material.

Materials and Methods

N. meningitidis strains

Strains used as a target of the bactericidal activity and their characteristics are listed in Table 1. Every strain was kept in Greaves solution to -70°C.

Serogroup	Strain	Classification Phenotype
A	F8238	A:4,21:P1.20,9:L11
	MK499/03	A:4,21:P1.20,9
B	Cu385/83	B:4,7:P1.19,15:L3,7,9
	NZ228/98	B:4:P1.7-2,4
C	C11	C:2a:P1.2,5:L3, 7, 9

Table 1: Strains of *N. meningitidis*.

Monoclonal Antibodies (MAbs) against *N. meningitidis*: MAbs were obtained at IFV in the Monoclonal Antibodies Department by the hybridoma method [10,11,3]. In this study, seven MAbs, specific to Outer Membrane Proteins (OMP) and polysaccharides (Ps) were employed; all of them murine and listed in Table 2. These MAbs were tested to determine their capacity to activate the complement system in human serum. All of them were used at a starting concentration of 1 mg/ml.

MAbs	Class/Subclass	Ag specificity
7E1F7	IgG2a	Ps-A
CU-NmPorA15	IgG2b	P1.15
CUNm5c	IgG2b	OpcA
7E12B3	IgG1	Ps-C
CU-NmPorA4	IgG1	P1.4
CUNmPorB4	IgG1	B4
CUNmFrpB	IgG1	FrpB

Table 2: Murine MAbs obtained at IFV [10,11,3].

Whole cell ELISA

Inactivated cell suspensions in PBS (Phosphate Buffer Solution) were adjusted to give an absorbance of about 0.1 at 600nm wavelength. The bacteria were dried onto the wells of 96 wells plates (Costar) at 37°C for 18-24 h. After three washes with PBS-Tweens, the plates were blocked with 3% (w/v) skimmed milk powder in PBS for 30min at 37°C. MAbs were added at 10 µg/mL and incubated at 4°C for 18-24 h. MAbs against OMP were evaluated against every strain and anti-Ps MAbs were evaluated against their homologous strains. Plates were washed and incubated with peroxidase-conjugated anti-mouse IgG diluted 1/10000 (Sigma) and incubated for 1 h at room temperature. The plates were developed at room temperature with substrate solution (12.5 mL of phosphate citrate buffer pH 4.6, 5 mg of 1,2 o-phenylenediamine (OPD) and 5 µL de H_2O_2 (Merck). Finally, the reaction was stopped by adding 50 µL H_2SO_4 (1%, v/v) and the OD492 nm read with a Multiskan Plus Microplate Reader (Labsystem).

In this test, an anti-meningococcal serogroup A and C Monoclonal Antibody (NIBSC, 95/674 and 95/678) was used as a positive control for strains of serogroup A and C, respectively.

SBA: the method used in this work was described by Cedré et al. (2012). Two strains of serogroup A, two of serogroup B and one of serogroup C were used. Cellular suspensions in HBSS (Hank s' Balanced Salt Solution from Sigma) were prepared from a 4h culture and adjusted to 8 x 104 CFU/mL.The complement source was baby rabbit serum (Peel Freez, MO). Equal volumes (10 µL) of the bacterial suspension and complement were added to 20 µl of monoclonal antibodies two-fold serial dilutions. The reaction mixture was then incubated at 37°C for 60 min and 10 µL was removed to a CBA (Columbia Blood Agar) plate by the tilt method. SBA titers were expressed as the reciprocal of the final serum dilution giving 50% killing, compared to the number of CFU in the suspension control. In this assay seven MAbs were evaluated against the respective strains.

As a control of this assay, we used for serogroup A and C, a commercial polyclonal human serum (NIBSC 99/706, ELISA standard), and for serogroup B, an in-house serum control from a mouse immunized intramuscular with VA-MENGOC-BC®.

Results and Discussion

Whole cell ELISA

The specificity of each MAb against the strains was evaluated using a whole-cell ELISA.

MAbs that recognised the cell surface were selected on the basis of optical densities five times greater than the blank (PBS).The positive MAbs/cell reaction confirmed the specificity for the epitopes, externally exposed in the cell membrane for MAbs against OMP or Ps.

Figure 1 shows that both MAbs against polysaccharide capsule (7E1F7 and 7E12B3) recognise strains from their homologous serogroups (A and C) as was described by Reyes et al. 2013. These authors demonstrated that 7E1F7 and 7E12B3 MAbs were suitable to identify specific Ps in commercial meningococcal multivalent vaccines MENCEVAX® ACWY, MENVEO® or vax-MEN-ACW135® by an indirect ELISA assay [3].

On the other hand, anti-OMP MAbs, CUNm5c and CUNmFrpB, did not recognize the Opca and FrpB proteins on the cell surface (Figure 1), even when these proteins are strongly preserved among *N. meningitidis* strains. It probably occurs because the epitopes recognized by MAbs are not exposed to the external cell surface and therefore are not accessible using the whole cell ELISA technique.

Figure 1 also shows that MAbs against PorA, CU-NmPorA15 and CU-NmPorA4, recognised Nm-B CU385 and Nm-B NZ228 respectively, and PorB (CUNmPorB4) recognized both strains from the serogroup B and strains from serogroup A. These results are in agreement with the phenotypic characteristics of strains shown in Table 1. Besides, the recognition of MAb against PorA to the whole cell was previously confirmed by a whole cell ELISA with the same strains by Perez et al [10].

Finally, MAbs 7E12B3, 7E1F7, CUNmPorB4, CU-NmPorA15 and CU-NmPorA4 were selected for evaluation in the bactericidal assay. For the SBA we used MAb 7E12B3 for serogroup C; 7E1F7 and CU-NmPorB4 for the two strains of serogroup A (F8238 and MK499/03);

CU-NmPorA15 and CUNmPorB4 for serogroup BCu385/83 strain, and CU-NmPorA4 and CUNmPorB4 for serogroup BNZ228/98 strain.

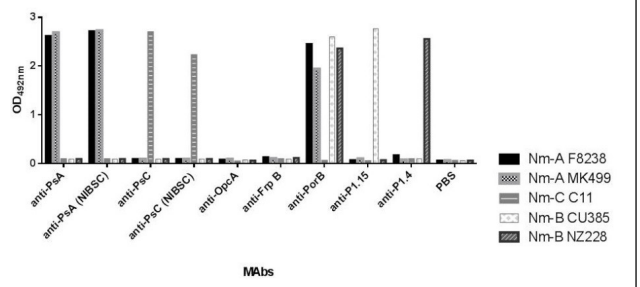

Figure 1: Recognition by the whole-cells ELISA of each MAbs to all *N. meningitidis* strains in this study.

Serum bactericidal assay (SBA)

In the study carried out to determine the presence of bacterial activity of murine MAbs, three of them showed bactericidal activity: anti-PsA (7E1F7), anti-P1.15 (CU-NmPorA15) and anti-P1.4 (CU-NmPorA4) (Figure 2).

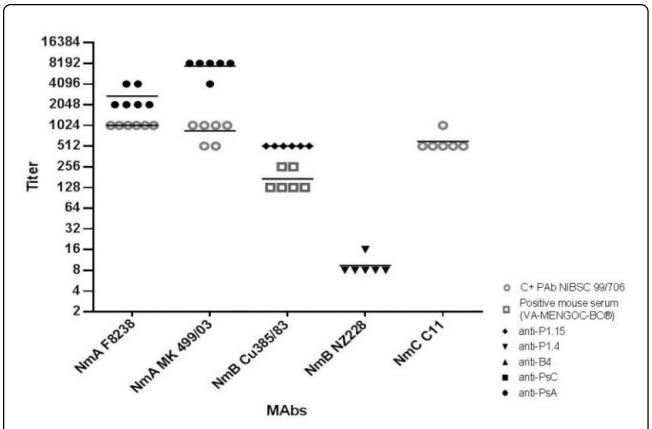

Figure 2: SBA titers of selected MAbs against *Neisseria meningitidis* strains (F8238, MK499/03, Cu 385/83, NZ228 y C11).

MAb anti-PsA showed higher serum bactericidal titers for both homologous strains (F8238 and MK499/03), even higher than the NIBSC positive control for ELISA. The MAb anti-PsC (7E12B3) showed no bactericidal activity, while anti-PorA MAbs (P1.4 and P1.15) showed lytic activity for NZ228/98 and CU385/83 strains, respectively. The serum bactericidal titer elicited by anti-P1.15 was higher than the in house positive serum.

Based on the results, and taking into consideration the differences between MAb subclasses and their capacity to activate complement; it was possible to demonstrate a relationship between the IgG subclass and bactericidal activity. It has been described in previous studies that the development of bactericidal activity depends on the activation of the classic pathway of complement [12]. Seino et al. proposed that the activation of the classical and alternative pathways by MAbs of the same isotype do not necessarily activate human complement to the same extent. Although, they demonstrated that mouse IgG1, IgG2 and IgM isotype are able to activate both pathways of human complement, but to activate the classic pathway (CP) they found that mouse IgM and IgG2 are better activators of this pathway than IgG1 isotype. In addition, they showed that IgG2a and IgG2b are potent activators of the CP; however, they found six of seventeen IgG1 activators of the CP of complement [13-15]. These results can explain the differences we obtained with the panel of MAbs and their functionality in SBA. Also these results encourage the production of MAbs against bacterial antigens and the selection of clones from IgG2 isotypes (IgG2a and IgG2b) to be used in biological functional assays as a positive control. In addition, the MAbs obtained, could work as positive control in the immunological evaluation of vaccines by ELISA.

Conclusions

Three monoclonal antibodies were identified with bactericidal activity against strains of *N. meningitidis* and will be used as controls in the SBA to validate the assay and verify the quality of vaccine lot produced at IFV against meningococcal disease.

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