Development and characterization of murine monoclonal antibody specific for the P1.4 PorA proteins from strain B:4:P1.(7b).4. of *Neisseria meningitidis*

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Neisseria meningitidis isolates are conventionally classified by serosubtyping that characterizes the reactivities of the PorA outer membrane protein variable-region epitopes with monoclonal antibodies. Porins are outer membrane proteins (OMPs) of *N. meningitidis* serogroup B and have attracted study principally for two reasons: their use in the classification of meningococcal isolates into serotype and subtype and as potential components of vaccines against this important pathogen. New murine hybridomas, secreting specific monoclonal antibodies against PorA serotype P1.4 of *N. meningitidis* serogroup B, were generated using conventional hybridoma procedures. The monoclonal antibodies obtained were characterized by Western blot and whole cell ELISA, using reference strains from different *N. meningitidis* serotypes and subtypes. All monoclonal antibodies belong to isotype IgG1. Others hybridomas producing MAbs against PorB and FrpB were also obtained.

Keywords: Monoclonal Antibodies, PorA, *Neisseria meningitidis*.

Introduction

The obligate human pathogen *Neisseria meningitidis* is a Gram-negative bacterium that variably colonizes the nasopharynx of healthy individual (1, 2). Invasive meningococcal disease causes a significant public health burden throughout the world, with estimates of 500 000 cases and more that 50 000 deaths reported annually (3).

The inability to use a polysaccharide vaccine to protect against group B meningococci has limited the ability to control group B meningococcal disease by vaccination. The weak immunogenicity of the group B polysaccharide means that subcapsular antigens, and in particular the PorA and PorB outer membrane proteins, have become a focus as vaccine components.

The class 1 protein is PorA. Expression of the class 2 and class 3 proteins (PorB) is mutually exclusive, and they are products of the *porB* locus. PorA and PorB are important epidemiological markers that are the targets of serosubtyping and serotyping antibodies, respectively and are the targets of bactericidal antibodies (4).

Since mid-1991, New Zealand has experienced an epidemic of meningococcal disease. The epidemic has been caused by serogroup B meningococci expressing PorA type P1.7-2, 4, belonging to the ST-41/ST-44 complex, lineage III. In various western countries, subtype P1.4 of *Neisseria meningitidis* serogroup B causes the greatest incidence of meningococcal disease too (5).

We report the generation of hybridomas producing monoclonal antibodies (MAbs) that recognized PorA outer membrane protein only from *N. meningitidis* strains subtype P1.4.

Materials and Methods

N. meningitidis outer membrane vesicles

Outer membrane vesicles (OMV) were manufactured by a detergent extraction method (6). Briefly: OMVs were obtained from live bacteria by gentle extraction with 10% deoxycholate (Merck). Bacterial debris was removed by centrifugation and nucleic acids were eliminated by enzymatic treatment with nucleases (Merck). OMVs were purified by gel filtration chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals) followed by precipitation with 96% ethanol. The outer membrane vesicles from B:4:P1.(7b).4 strain were used for immunizing the BALB/c mice and ELISA.

Bacterial strains, growth condition and monoclonal antibodies

Prototype strains used in this work were stored at -70 ºC in 10% skim milk (Oxoid, England) containing 20% (v/v) glycerol; before use they were cultured on GC (Difco) agar plate overnight in a humid atmosphere containing 5% CO_2. The bacteria were then scraped from the plate with sterile cotton swabs and suspended in phosphate-buffered saline (PBS).

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After inactivation of bacteria at 56 ^{0}C in a water bath for a minimum of 30 minutes, the suspension was adjusted to an absorbance of 0.09 or less, and then stored at 4 ^{0}C until used. Specific P1.4 subtype MAb (MN20B9.34, IgG2a) was used as control, and it was supplied by Pierre Voet from GSK.

Enzyme immunoassay for detection of specific antibodies

Polystyrene micro titer ELISA plates (Costar, USA) were coated overnight at 4 ^{0}C with 100 mL per well of OMV a concentration of 5 mg/mL in PBS. The wells were subsequently filled with 2% skim milk solution in PBS and incubated for 1 h at room temperature (RT) to reduce non-specific binding. Samples, consisting in serial dilution of sera or culture supernatants, were added and incubated for 2 h at RT. Plates were washed with 0.05% tween-20 in PBS (PBS-T) and incubated with peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co., USA). Color was developed with o-phenylenediamine (0.4 mg/mL) and 0.4% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0. The reaction was stopped by adding 2.5 N H_2SO_4 and the O.D.$_{492nm}$ read with a plus-multiscan microplate reader (Labsystem, UK).

Whole-cell enzyme immunoassay

Several suspensions of *N. meningitidis* strains were used for coating the well of polystyrene microtiter ELISA plates (Costar, USA) overnight at 37 ^{0}C. Samples, consisting in purified monoclonal antibodies or culture supernatants were added and incubated for 2 h at RT. Plates were washed with PBS-T and incubated with peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co., USA). Color was developed with o-phenylenediamine (0.4 mg/mL) and 0.4% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0. The reaction was stopped by adding 2.5 N H_2SO_4 and the O.D.$_{492nm}$ read with a plus-multiscan microplate reader (Labsystem, UK).

Production of MAbs

Female BALB/c mice (6-8 week old) were immunized by SC injection of OMV (20 μg) emulsified in Freund's adjuvant (Sigma Chemical Co., USA). Each animal received four injections administrated at 2-week intervals. Mice were bled by their tail veins 7 to 10 days after the final injection and their serum tested for anti-OMV antibodies in the ELISA

described above. Three to four days before cell fusion, the appropriate mouse received a final injection of antigen (10 mg) in PBS. Splenocytes were fused with the P_3X_{63}-Ag 8.653 mouse myeloma cell line using polyethylene glycol 1300 Hybri-Max (Sigma Chemical Co., USA) as described by Campbell (7). The hybrid cells were screened for their ability to secrete antibodies binding OMV in the direct ELISA (350 clones assay) and 200 clones for Western blot.

A second round of screening was made by Western blot analysis using as whole-cell and a reference MAb specific to subtype P1.4. Hybrids-secreting reactive antibodies were subcloned by limiting dilution and stabilized. The selected hybridoma cells were grown as ascites in the peritoneal cavity of pristane-primed BALB/c mice. Ascites fluid was tapped from the peritoneal cavity and rendered cell-free by centrifugation at 1000 g for 15 min at 4 ^{0}C. The MAbs were purified from ascites fluid using protein A affinity chromatography (8).

Isotyping

Classes and subclasses of MAbs secreted by hybridoma were determined with an ImmunoType Kit (Sigma Chemical Co., USA) following manufacturer's instructions.

Immunoblot analysis of MAbs

OMV or whole-cell lysates were fractionated in 12.5% SDS-PAGE according to Laemmli (9) and transferred to nitrocellulose paper as described by Towbin (10). After blocking with 5% skim milk in PBS, the blot was reacted with a suitable dilution of MAbs. The papers were washed with PBS-T and incubated with peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co., USA). Color was developed with diaminobenzidine (0.2 mg/mL) (Sigma Chemical Co., USA) and 0.4% H_2O_2 in tris-buffered saline, pH 8.0.

Results

Hybrids producing anti-OMV antibodies were screening by a direct ELISA and Immunoblotting. Table 1 shows the results of specific MAb-secreting clones obtained against different antigens (PorA, PorB and FrpB).

Table 1. Specificity of the Monoclonal antibodies obtained using Immunoblot and ELISA techniques in the primary screening.

Specific MAbs	Primary screening		Total MAbs
	Immunobloting	ELISA	
PorA	2	2	4
FrpB	6	-	6
PorB3	6	6	12
No. clones (+)	14	9	23
No. clones analyzed	200	350	

Immunoblot analysis and ELISA of MAb anti PorA

The specificity of the obtained MAbs anti PorA were studied by Immunoblot analysis using a strain of *N. meningitidis* separated by SDS-PAGE. Figure 1 (lane 1-4) shows that all MAbs recognized a 46-kDa antigen, corresponding to PorA protein from *N. meningitidis* serogroup B strain B:4:P1.(7b).4. This finding was corroborated by using reference MAbs anti PorA protein against subtype P1.4. The four MAbs obtained were coded as follow: CU-NmPorA4 (54); CU-NmPorA4(80); CU-NmPorA4(324) and CU-NmPorA4(447). The isotype of all MAbs were IgG1.

Figure 1. The specificity of the MAbs were analyzed by Western blot using OMV from B:4:P1.(7).4 strains. Lanes 1-4 corresponding to MAbs anti Por A obtained (CU-NmPorA4(54), CU-NmPorA4(80), CU-NmPorA4(324), CU-NmPorA4(447) and line 5 corresponding to referent MAb (MN20B9.34).

We also evaluated the specificity for subtypes P1 (7) and P1.4, corresponding to RV1 and RV2, respectively. To do this we tested the MAbs anti PorA in whole-cell enzyme immunoassay and Immunoblot using a panel of different serotype and subtype *N. meningitidis* strains. The MAbs only recognized the strains that included in their antigenic structure P1.4 protein as shown in Table 2 and Figure 2 (A, B, C, D). The results were endorsed by using anti P1.4 specific MAb in both techniques.

Discussion

Methods, which use a 96-well configuration (ELISA), are clearly the most practical tool for screening large number of wells required for monoclonal antibody production. Here a double-screening method (Immunoblot and ELISA) was used in the primary screening in order to identify positive clones. The decision to use Immunoblotting as screening strategy in hybridoma production against OMV proteins was based on several factors: the antigen preparation used for both immunization and screening was OMV; the inaccessible P1.7 subtype epitope from PorA protein only is available after protein denaturalization (11); previous findings showed that when OMV from CU-385-83 strains was used for MAbs generation only was obtained MAbs against PorA and PorB3 proteins (12); and finally sera collected from mice immunized with OMV protein and examined by Immunoblotting recognized several different antigens, including PorA, PorB3 and FrpB, among others.

As it is shown in the Table 1, MAbs against to FrpB only were detected when using Immunobloting assay as the selection methods for primary screening, whereas MAbs against PorA and PorB were detected using both Immunobloting assay and ELISA. MAbs against PorA were analyzed in more detail later and all of them reacted in whole cell ELISA and Immunoblotting only against subtype P1.4 strains.

The use of this screening strategies contributed to obtain MAbs against FrpB, PorA subtype P1.4 and PorB3 proteins from outer membrane of *Neisseria meningitidis*. The reason for the inaccessibility of the epitope from FprB in ELISA assay using OMV as coating antigen, are now being studied.

Characterization of meningococcal strains has been based on antigenic differences in the capsule (serogroup), the four variable regions (VRs) of the PorB OMP (serotype), and the two variable regions (VRs) of the PorA OMP (serosubtype)
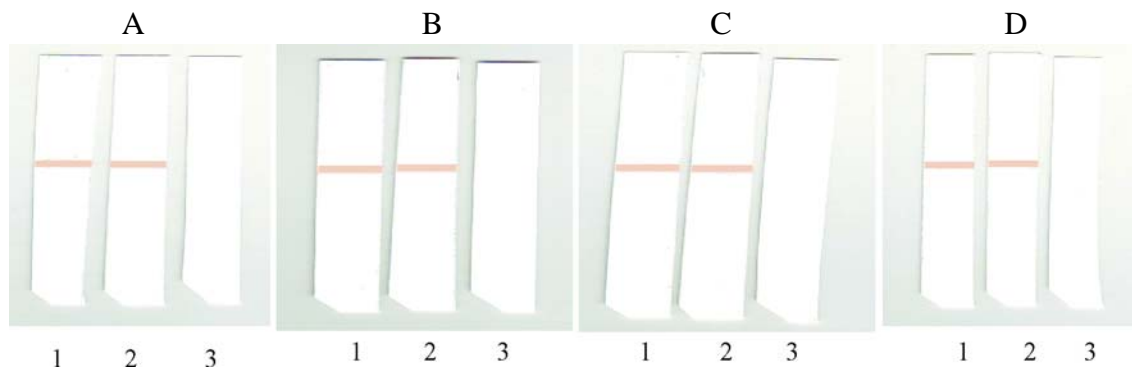
Table 2. Reactivity of Mabs anti PorA (CU-NmPorA4(54), CU-NmPorA4(80), CU-NmPorA4(324) and CU-NmPorA4(447) against several *N. meningitidis* strains, determined by whole cell ELISA. Referent MAbs anti P1.4 subtype (MN20B9.34) used as positive control.

Strains Total		MAbs under analyze anti PorA				MN20B9.34
		CU-NmPorA4(54)	CU-NmPorA4(80)	CU-NmPorA4(324)	CU-NmPorA4(447)	
B:4:P1(7).4	25	+	+	+	+	+
B:4:P1.19.15	10	-	-	-	-	-
B:NT:P1.4	1	+	+	+	+	+
B:4:P1.4	1	+	+	+	+	+
B:1:P1(7)	1	-	-	-	-	-
B:15:P1(7).16	1	-	-	-	-	-
B:2a:P1.2	1	-	-	-	-	-
B:14:P1.6.14	1	-	-	-	-	-
B:4:P1.10	1	-	-	-	-	-
NA:NT:P1.4	2	+	+	+	+	+

Figure 2. Immunoblot analysis for the subtype specificity P1(7) and P1.4. The lanes contained the following strains: lane 1 (228) P1.(7b).4 strain; lane 2 (882066) P1.4 strain; lane 3 (M1080) P1.7 strain. Blocks A, B, C and D showed the reactivity of to CU-NmPorA4(54), CU-NmPorA4(80), CU-NmPorA4(324) and CU-NmPorA4(447) MAbs, respectively.

(13). This last one is being considered a promising vaccine candidate (14).

Monomeric PorA is a transmembrane protein with eight outer loops, among which the first and the fourth loop contain the hypervariable regions, called VR1 and VR2, respectively (15). These two regions define the dual PorA subtype, designated P1. x, y, where "P1" stands for the class 1 protein and "x" and "y" stand for numbers denoting the VR1 and VR2 domains, respectively. The two subtype regions from PorA are generally determined by an whole cells ELISA or a blotting assay (16) using referent MAbs directed against epitopes in VR1 and VR2; consequently, each PorA can bind two different subtype specific MAbs.

We have produced and characterized four MAbs against PorA outer membrane protein only from *N. meningitidis* strains subtype P1.4. Western blot analysis (Figure 1) showed that MAbs anti PorA recognized an approximately 46 kDa protein presented in *N. meningitidis* B:4:P1.(7b).4. strain, that closely corresponds to the molecular mass predicted for the product of the *Por A* gene (16).

The antibodies only reacted with subtype P1.4 strains when it was evaluated by Immunoblot analysis and whole cell ELISA (see Figure 2 A, B, C, D and Table 2). These results suggested that our MAbs were specific for VR2 of PorA protein.

The most appropriate screening methods and subtyping analysis are required for an adequate investigation. A panel of well-characterized subtype-specific MAbs should be selected, in order to classify more strains.

The production of new MAbs will improve and facilitate the study of the structure and function of different *N. meningitidis* antigens in great detail, permitting to carry out other characterization studies from clinical isolates, necessary to increase the epidemiological knowledge.

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Desarrollo y caracterización de un anticuerpo monoclonal murino específico a la proteína PorA P1.4 de la cepa B:4:P1.(7b).4 de *Neisseria meningitidis*

Resumen

Los aislamientos de *Neisseria meningitidis* se clasifican convencionalmente por serosubtipos. Su reactividad se realiza entre el epítope de la región variable de la proteína de membrana externa PorA con anticuerpos monoclonales. Las porinas, proteínas de membrana externa de *N. meningitidis* del serogrupo B, son atractivas para su estudio principalmente por la clasificación en serotipo y subtipo de los aislamientos del meningococo y como posibles componentes de vacunas contra este importante agente patógeno. Se generaron nuevos hibridomas murinos secretores de anticuerpos monoclonales específicos contra la proteína PorA subtipo P1.4 de *N. meningitidis* del serogrupo B, mediante los procedimientos convencionales de hibridomas. Los anticuerpos monoclonales, pertenecientes al isotipo IgG1, fueron caracterizados mediante Western blot y ELISA de células enteras. Se utilizaron cepas de referencia de diferentes serotipos y subtipos de *N. meningitidis* y se obtuvieron hibridomas productores de anticuerpos monoclonales contra otras proteínas como PorB y FrpB.

Palabras clave: Anticuerpos monoclonales, PorA, *Neisseria meningitidis.*