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Development and Characterization of a Murine Monoclonal Antibody Specific for the P1.15 PorA Proteins from Vaccine Strain B:4,7:P1.19,15 of *Neisseria meningitidis*

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ABSTRACT

***Neisseria meningitidis* isolates are conventionally classified by serosubtyping, which characterizes the reactivities of the PorA outer membrane protein variable-region epitopes with monoclonal antibodies (MAbs). New murine hybridomas, secreting specific MAbs against PorA of *N. meningitidis* serogroup B, were generated using conventional hybridoma procedures. Using outer membrane protein as antigen, we obtained two positive clones, and one of them we characterized. This MAb reacted, on whole-cell enzyme-linked immunosorbent assay (ELISA) and immunoblotting, only with strain subtype P1.15 and its IgG2b isotype. This MAb demonstrated bactericidal activity against the homologous strain in the presence of human complement.**

INTRODUCTION

NEISSERIA MENINGITIDIS is an important cause of morbidity and mortality worldwide, and the leading cause of bacterial meningitis and septicemia in children and young adults.[1]

Traditionally, strains were characterized by using antibodies that recognized surface exposed epitopes on the capsule or outer membrane.[2] By this technique, 13 serogroups, 20 serotypes, and 29 subtypes have been defined. Twelve immunotypes have been defined by lipopolysacharides.[3] An example of serological typing nomenclature is B:4,7:P1.19.15, indicating that it is meningococci serogroup B, serotype 4,7, and subtype P1.19.15.

Porins are outer membrane proteins (OMPs) of *N. meningitidis* serogroup B and have attracted study principally for two reasons: (i) their use in the classification of meningococcal isolates into serotype and subtype, and (ii) as potential components of vaccines against this important pathogen.[4]

Class 1 OMPs have been named PorA and its gene designated *por A*.[5] Similarly, class 2 and class 3 OMPs have been named PorB (PorB2 and PorB3). The antigenic varieties of meningococcal PorA and PorB proteins have been used for serotyping and serosubtyping, respectively.[6]

We report the generation of one hybridoma producing monoclonal antibody (MAb) that recognized PorA outer membrane protein only from *N. meningitidis* strains subtype P1.15.

METHODS

Outer membrane vesicles of N. meningitidis

The active pharmaceutical ingredients of Cuban OMV vaccine VA-MENGOC-BC® (Finlay Institute, Cuba) were used for immunizing BALB/c mice and for conducting enzyme-linked immunosorbent assay (ELISA). Outer membrane vesicles (OMVs) were manufactured from the outer membranes of *N. meningitidis* serogroup B strain CU-385-83 (B:4,7:P1.19,15; L3,7,9) by a detergent extraction method.[7] Briefly, OMVs were obtained from live bacteria by gentle extraction with 10% deoxycholate (Merck, Franklin Lakes, NJ). 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, Sweden) followed by precipitation with 96% ethanol.

Bacterial strains, growth conditions, and monoclonal antibodies

Prototype strains used in this work were conserved at −70°C in 10% skim milk (Oxoid, UK) 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 bac-

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teria were then scraped from the plate with sterile cotton swabs and suspended in phosphate-buffered saline (PBS). After inactivation of bacteria at 56°C in a water bath for a minimum of 30 min, the suspension was adjusted to an absorbance of 0.09 or less, then stored at 4°C until use.

The MAbs (MN3C5C anti–subtype P1.15; 2-1-P1.19 anti–subtype P1.19) directed against PorA protein have been described previously and are reference antibodies for determination of subtype specificity.[4,8]

Enzyme-linked immunosorbent assay for detection of specific antibodies

Polystyrene microtiter enzyme-linked immunosorbent assay (ELISA) plates (Costar) were coated overnight at 4°C with 100 μL per well of OMV at a concentration of 5 μg/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, 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 immunoglobulin G (IgG; whole molecule; Sigma, St. Louis, MO). 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 OD_{492nm} read with a plus-multiscan microplate reader (Labsystem, UK).

Production of monoclonal antibodies

Female BALB/c mice (6–8 weeks old) were immunized by subcutaneous injection of OMV (20 μg) emulsified in Freund's adjuvant (Sigma). Each animal received four injections administrated at 2-week intervals (the first immunization with Freund's complete adjuvant and the rest with Freund's incomplete adjuvant). Mice were bled by their tail veins 7–10 days after the final injection, and their serum was tested for anti-OMV antibodies in the ELISA described above. At 3–4 days before cell fusion, the appropriate mouse received a final injection of antigen (10 μg) in PBS. Splenocytes were fused with the P_3X_{63}-Ag 8.653 mouse myeloma cell line using polyethylene glycol (PEG) 1300 Hybri-Max (Sigma) as described by Campbell.[9] The hybrid cells were screened for their ability to secrete antibodies binding OMV in the direct ELISA. A second round of screening was made by Western blot analysis using as antigens OMV and a reference MAb specific to OMP subtype P1.15. Hybrids-secreting reactive antibodies were subcloned by limiting dilution and stabilized. One clone, coded CU-NmPorA15(212), was isolated and characterized. 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 $\times$1,000*g* for 15 min at 4°C. The MAbs were purified from ascites fluid using protein A affinity chromatography.[10]

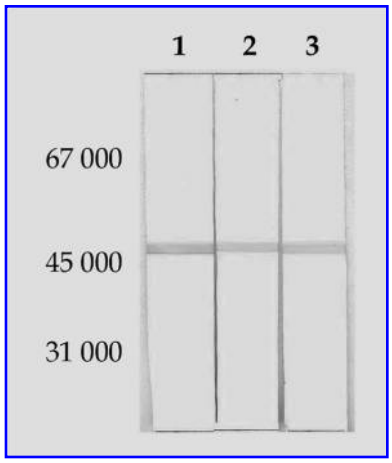

FIG. 1. Immunoblot analysis of outer membrane vesicle (OMV) from *Neisseria meningitidis* strain CU-385-83 separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The antibodies used for detection were lane 1, monoclonal antibody (MAb) CU-NmPorA15 (212); lane 2, MAb MN3C5C (control PorA subtype P1.15-specific MAb); and lane 3, MAb 2-1-P1.19 (control PorA subtype P1.19-specific MAb). Sample was boiled in the presence of SDS and β-mercapto-ethanol. A broad-range molecular weight standard kit (BioRad, CA) was run together with samples and molecular weights, indicated in daltons (Da).

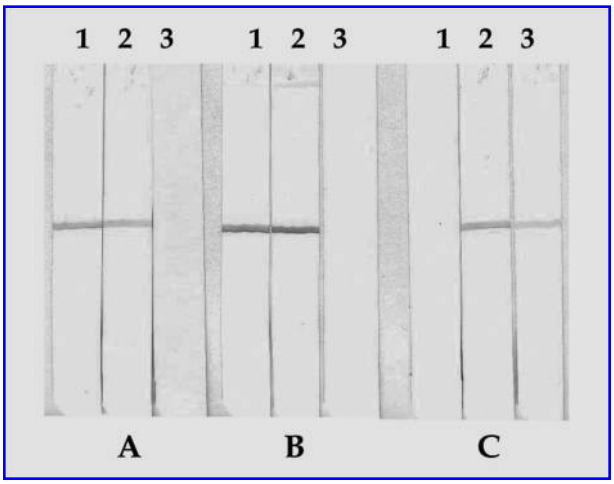

FIG. 2. Immunoblot analysis of *Neisseria meningitidis* cell lysates with monoclonal antibody (MAb) CU-NmPorA15 (212) (**A**); MAb MN3C5C (control PorA subtype P1.15-specific MAb) (**B**); MAb 2-1-P1.19 (control PorA subtype P1.19-specific MAb) (**C**). Lane 1, 305/95 (B: 4: P1.7,15); lane 2, CU-385-83 (B: 4,7: P1.19,15); lane 3, 19/92 (B: 4: P1.19,2c). Sample was boiled in the presence of sodium dodecyl sulfate (SDS) and β-mercapto-ethanol.

Isotyping

The classes and subclasses of MAbs secreted by hybridoma were determined with an ImmunoType Kit (Sigma) following the manufacturer's instructions.

Immunoblot analysis of monoclonal antibodies

OMV or whole-cell lysates were fractionated in 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli[11] and transferred to nitrocellulose paper as described by Towbin.[12] After blocking with 5% skim milk in PBS, the blot was reacted with a suitable dilution of MAbs. The

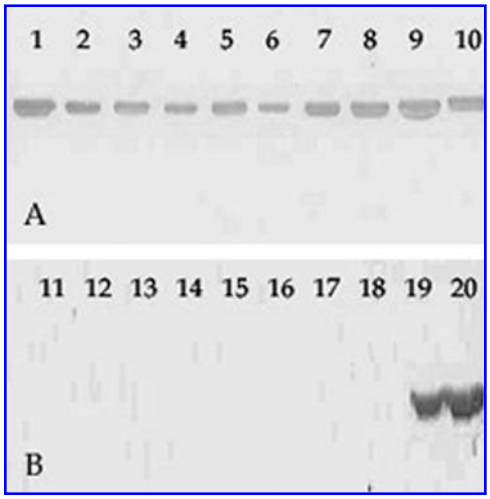

FIG. 3. Immunoblot analysis of *Neisseria meningitidis* cell lysates with monoclonal antibody (MAb) CU-NmPorA15 (212). (**A**) Subtype P1.19.15 strains: lane 1, CU-385-83; lane 2, 7/99; lane 3, 8/99; lane 4, 9/99; lane 5, 12/99; lane 6, 14008; lane 7, 14030; lane 8, 14031; lane 9, 14039; lane 10, H355. (**B**) No subtype P1.19.15 strains (except lanes 19 and 20): lane 11, 2996; lane 12, B16B6; lane 13, M990; lane 14, M1080; lane 15, M982; lane 16, 870227; lane 17; S3446; lane 18, H44/76; lane 19, H355; lane 20, CU-385-83. Sample was boiled in the presence of sodium dodecyl sulfate (SDS) and β-mercapto-ethanol. A broad-range molecular weight standard kit (BioRad, CA) was run together with samples and molecular weights, indicated in daltons (Da).

papers were washed with PBS-T and incubated with peroxidase-conjugated anti-mouse IgG (whole molecule; Sigma). Color was developed with diaminobenzidine (0.2 mg/mL; Sigma) and 0.4% H_2O_2 in TRIS-buffered saline, pH 8.0.

Whole-cell enzyme immunoassay

Several suspensions of *N. meningitidis* strains were used for coating the wells of polystyrene microtiter ELISA plates (Costar) overnight at 37°C. Samples consisting of purified MAbs 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). 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 OD_{492nm} read with a plus-multiscan microplate reader (Labsystem, UK).

Bactericidal assay

The bactericidal activity of the specific MAb was determined in sterile 96-well flat-bottom microtiter plates. Bactericidal activity of antibody purified was tested against *N. meningitidis* strains CU-385-83 in the presence of human complement as described previously.[13]

RESULTS

Production of monoclonal antibodies

Hybrids producing anti-OMV antibodies were screened by a direct ELISA, and one of them was selected on the basis of its reactivity against PorA by immunoblot analysis (Fig. 1). This hybridoma and its MAb was coded CU-NmPorA15 (212), which secreted antibodies of the IgG2b isotype. This MAb was purified from ascites fluid by protein A affinity chromatography. The approximate quantity of MAb secreted by this clone, as estimated from the yield of the purification process, was 4.5 mg/mL. Other hybridomas producing MAbs against PorB were also obtained (data not shown).

Table 1. Reactivity of Monoclonal Antibody (MAb) CU-NmPorA15 (212) with a Variety of Bacterial Strains as Determined by Whole Cell Enzyme-Linked Immunosorbent Assay (ELISA)

		Monoclonal antibodies		
Serological typin characteristics	*No. of strains*	*CU-NmPorA15 (212)*	*MN3C5C*	*2-1-P1.19*
B: 4,7: P1.19,15[a]	1	+	+	+
B:4: P1.19,15	8	+	+	+
B:15: P1.19,15	1	+	+	+
B:2B: P1.5,2	1	−	−	−
B:2a: P1.5,2	1	−	−	−
B:16,6: P1.6	1	−	−	−
B:19,7,1: P1.7,1	1	−	−	−
NG: P1.22,9	1	−	−	−
B:4,7: P1.10	1	−	−	−
B:19,14: P1.23,14	1	−	−	−
B:15: P1.7,16	1	−	−	−
B:4:P1.19,2c	1	−	−	+
B:1:P1,2,19	1	−	−	+
B4:P1.14,19	1	−	−	+
B:4:P1.7,15	1	+	+	−

[a]Strain CU-385-83.
NG, nongroupable.

Immunoblot analysis of the monoclonal antibody

The specificity of the obtained MAb was studied by immunoblot analysis of OMV from *N. meningitidis* strain CU-385-83 separated by SDS-PAGE. Figure 1 (lane 1) shows that MAb CU-NmPorA15 (212) recognized a 46-kDa antigen, corresponding to the PorA protein from *N. meningitidis* serogroup B strain CU-385-83. This finding was corroborated by using two reference anti–PorA protein MAbs (MN3C5C and 2-1-P1.19) against subtype P1.15 and P1.19, respectively. These MAbs recognized the same protein band as the MAb obtained by us (Fig. 1, lanes 2 and 3).

We also evaluated the specificity of MAb CU-NmPorA15 (212) against *N. meningitidis* cell lysates in immunoblot analysis. Figure 2A shows that our MAb only recognized a protein band in the P1.15 but not P1.19 strain. Figure 2B,C represents the results using specific anti–P1.15 and anti–P1.19 MAbs against the same group of strains.

Furthermore, the anti–P1.15 specificity of MAb CU-NmPorA15 (212) was demonstrated by immunoblot analysis and whole-cell ELISA using a panel of different serotype and subtype *N. meningitidis* strains. The MAb only recognized the strains that included, in their antigenic structure, the P1.15 protein (Fig. 3 and Table 1). The results were substantiated by using anti–P1.15 and P1.19-specific MAbs in both techniques.

Finally, MAb CU-NmPorA15 (212) demonstrated bactericidal activity against the homologue strains in the presence of human complement.

DISCUSSION

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).[14]

PorA OMP of *N. meningitidis* has been useful as subtyping antigen for seroclassification of meningococci, and it is considered a promising vaccine candidate.[15,16]

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.[17,18] 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 of PorA are generally determined by whole-cell ELISA or blotting assay[18,19] 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 an MAb against PorA outer membrane protein only from *N. meningitidis* strains subtype P1.15. Western blot analysis (Fig. 1) showed that MAb CU-NmPorA15 (212) recognized an approximately 46 kDa protein produced by *N. meningitides* CU-385-83 strain that closely corresponds to the molecular mass predicted for the product of the *Por A* gene.[20,21] The antibody only reacted with subtype P1.15 strains when it was evaluated by immunoblot analysis and whole cell ELISA (Fig. 3 and Table 1). These results suggest that our MAb is only recognizing the VR2 of PorA protein. It is known that P1.19.15 subtype has been the most prevalent subtype in many countries for a long time,[22,23] so it would be very useful to count with a new P1.15 subtyping specific MAb for seroclasification of meningococci.

On the other hand, the MAb CU-NmPorA15 (212) demonstrated to have bactericidal activity against *N meningitides* CU-385-83 strain, reinforcing the idea that antibodies against PorA OMP of *N. meningitidis* may be involved in the immunological defense mechanism against meningococci *in vivo*.[24]

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

Finally, MAb CU-NmPorA15 (212) has been used successfully for several years in the identity assays to evaluate the active pharmaceutical ingredient of Cuban OMV vaccine VA-MENGOC-BC®.[25]

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