Expression of Class 5 Antigens by Meningococcal Strains Obtained From Patients in Brazil and Evaluation of Two New Monoclonal Antibodies

Elizabeth N. De Gaspari and Wendell D. Zollinger

Adolfo Lutz Institute, São Paulo, SP, Brazil
Walter Reed Army Institute, Maryland, USA

Determining the profile of antigen expression among meningococci is important for epidemiologic surveillance and vaccine development. To this end, two new mouse monoclonal antibodies (MAbs) have been derived against *Neisseria meningitidis* proteins (class 5). The MAbs were reactive against outer membrane antigens and were bactericidal. Selected anti-class 5 MAbs [(5.1)-3E6-2; (5.3)-3BH4-C7; (5.4)-1BG11-C7; (5.5)-3DH-F5G9 also 5F1F4-T3(5.c)], and the two new monoclonal antibodies C14F10Br2 (5.8) and 7F11B5Br3 (5.9), were then tested against different meningococcal strains, (63 strains of serogroup A, 60 strains of serogroup C (from 1972 to 1974); and 136 strains of serogroup B (from 1992) meningococci). Our results demonstrated that the expression of class 5 proteins in the *N. meningitidis* B Brazilian strains studied is highly heterogeneous. The serotypes and subtypes of B:4:P1.15, B:4:P1.9, B:4:P1.7, B:4:P1.3, B:4:P1.14, B:4:P1.16, B:4:NT, and B:NT:NT were detected in *N. meningitidis* B serogroups. The strains C:2a:P1.2 and A:4.21:P1.9 were dominant in the C and A serogroups, respectively. Serogroup B organisms expressed the class 5 epitopes 5.4 (18%), 5.5 (22%), 5.8 (3.6%), 5.9 (8.0%) and 5c (38%). Serogroup C expressed class 5 epitopes 5.1 (81%), 5.4 (35%), 5.5 (33%) and 5.9 (5.0%); and serogroup A showed reactivity directed at the class 5 protein 5c (47%); and reactivity was present with the new monoclonal antibody, 5.9 (5.5%). We conclude that the two new MAbs are useful in detecting important group B, class 5 antigens, and that a broad selection of serogroup B, class 5 proteins would be required for an effective vaccine based on the class 5 proteins.
Key Words: *Neisseria meningitidis*, monoclonal antibodies, outer membrane protein, Flow cytometry.

Meningococci have been classified into serogroups [1], based on their capsular polysaccharides. These serogroups differ in the characteristics of the diseases they cause. Meningococci of serogroup A are responsible for epidemics, particularly in sub-Saharan Africa [2] and China [3], whereas endemic disease and limited outbreaks in developed countries are mainly associated with serogroups B and C [4].

The incidence of meningococcal disease (MD) in Brazil has been monitored since the serogroup A and C epidemics that occurred between 1971 and 1974. In 1974, the number of cases exceeded 179 cases per 100,000 inhabitants [5,6]. The incidence of MD due to *N. meningitidis* serogroup C in the greater São Paulo area of Brazil, has been low since the end of the epidemics of 1971 and 1972. Between 1972 and 1974, there was an overlapping of two epidemic waves; one caused by serogroup C (91% of all MD cases in 1972), and another, larger wave caused by serogroup A (90% of all MD cases in 1974). Between 1981 and 1987, about 83% of the meningococcal isolates were identified as serogroup B, whereas only 6% corresponded to serogroup C strains. In 1988, the incidence of MD in the greater São Paulo area, state of São Paulo, was over 4 per 100,000 inhabitants.

It was caused by serogroup B strains in 1988 and 1989, and by serogroups B and C strains in 1990 [7]. During this period, the *N. meningitidis* serogroup B isolates obtained from MD cases were characterized by serological typing as B:4:P1.15, and by multilocus enzyme electrophoresis as belonging to 1 distinct cluster, included in the ET-5 complex of a group of 22 closely related ETs [8].

The major proteins of the outer membrane of *N. meningitidis* are designated class 1 through class 5 [10]. Class 1, 2, and 3 proteins are porins, and they show a limited antigenic variation that has been used to define serotypes and subtypes [11,12].

Class 5, or opacity (Opa), proteins are a family of antigenically variable outer membrane proteins of *N. meningitidis* [4]. These class 5 proteins are heat-modifiable and vary in size from 25 kD to 30 kD. Meningococci contain 4 *opa* genes that contain multiple repeats of the sequence CTCTT which results in phase variation of gene product expression due to slip-strand misparing which leads to a shift in the reading frame. As a result, the number of Opa proteins expressed varies from strain to strain [13-15]. The class 5 proteins encoded by opa genes (Opa proteins) have fairly constant sequences, except for 2 hypervariable (HV) regions [16-19]. Meningococci isolated on different continents, and defined by multilocus enzyme electrophoresis as belonging to the ET-37 complex, contain both shared and unique Opa HV regions encoding epitopes recognized by monoclonal antibodies (MAbs), and show evidence of recombinational reassortment of the HV regions [8, 9]. The class 5 protein called Opc or 5c is encoded by the *opc* gene and differs from Opa proteins [20,21] with which it shows only 22% amino acid sequence homology [22].

Bacteria isolated from the nasopharynx of patients and healthy carriers were found to express large amounts of 5c, whereas those isolated from cerebrospinal fluid or blood preferentially expressed small amounts of the protein [16]. This suggested that 5c might be important for adhesion to epithelial cells and that down-regulated variants might be selected during invasion.

The class 5 proteins have a basic isoelectric point, a trimeric structure, hypervariability of expression, heat modifiability and surface exposure. It should be pointed out that meningococcal class 5 proteins seem to induce strong strain-specific IgG antibody responses in humans after meningococcal disease and are also highly immunogenic in mice, where they elicit strain-specific bactericidal antibodies [22].

Until now, the frequency of most class 5 proteins expressed by Brazilian meningococcal strains had not been determined serologically. Here we present the results of such tests. This analysis is relevant for the development of a vaccine against this microorganism since it aids in allowing a careful selection of antigens for vaccine formulation. The preparation of 2 novel monoclonal antibodies specific for epitopes present in the class 5 protein derived from a Brazilian *N. meningitidis* epidemic strain, contribute to the determination of class 5 proteins to be included as antigens in the future vaccine for meningitis of serogroup B in Brazil. These MAbs are available for future use in class 5 evaluations in other countries.

Materials and Methods

Origin of the strains

All meningococci of serogroups A, B, or C, were isolated from the blood or cerebrospinal fluid specimens of patients with systemic disease and have been deposited in the strain collection of the Adolfo Lutz Institute. Following biochemical identification [23], the strains were serogrouped by the slide agglutination technique using antisera against the 9 major capsular serogroups of *N. meningitidis* in the Bacteriology Section of the Adolfo Lutz Institute. These antisera were prepared by the National Reference Center for Meningitis in the Immunology Section of the Adolfo Lutz Institute [24]. We analyzed 63 *N. meningitidis* strains of serogroup A, 60 strains of serogroup C (from the 1972-1974 epidemics) from the culture collection at the Adolfo Lutz Institute, and 136 strains of serogroup B (from 1992) obtained from the Bacteriology Section of the Adolfo Lutz Institute.

The bacteria were grown overnight in a candle jar on Tryptic Soy Broth (TSB; Difco BRL products, Gaithersburg, MD) supplemented with 1% horse serum (Sigma, St.Louis, MO) in plates in a 5% CO_2 atmosphere at 37°C. We confirmed the serogroups used in the present investigation using MAbs for capsular serogroups A (2D7-B-5), B (5C1-3H7), C (7H9-4), W (6G9-7) and Y (2C2-4.6).

Production of mouse monoclonal antibody

The 2 new monoclonal antibodies were obtained by fusion of mouse spleen cells with the murine myeloma line X63-Ag8.653 [25] as described [26]. Mice were immunized with 4 doses of meningococci B:4:P1.9 strain inactivated at 56°C, both intraperitoneally and intravenously, 3 days before fusion. Hybridomas were expanded and cultured in the peritoneal cavities of Pristane-primed mice to obtain ascitic fluid. All procedures with animals were in accordance with the principles of the Brazilian Code for the Use of Laboratory Animals. The anti-class 5 MAbs selected for this study were: 3E6-2 (5.1), 3BH4-C7 (5.3), 1BG11-C7 (5.4), 3DH-F5G9 (5.5), and 5F1F4-T3(5c), and the 2 new monoclonal antibodies C14F10Br2 (5.8) and 7F11B5Br3 (5.9). Except for the 2 novel ones (5.8) and (5.9), the remaining anti-class 5 monoclonal antibodies were prepared in the laboratory of one of the authors (WZ). The specificities of the 5.8 and 5.9 MAbs were determined by dot ELISA. The class and subclass of the 2 new MAb were determined by ELISA using conjugated anti-mouse immunoglobulins as described by the manufacturer (mouse hybridoma subtyping kits, Boehringer, Mannheim).

Preparation of outer membrane complex (OMC) and outer membrane protein (OMP) of N.meningitidis

OMC and OMP were prepared from the B:4:P1.9 Brazilian epidemic strain of *N. meningitidis*. Outer membrane components (OMC) were extracted in 5 mL of buffer solution (0.1 M sodium acetate and 0.2 M lithium chloride, pH 5.8) per g (wet weight) of cells by shaking with 2 mm diameter glass beads in a gyrating water bath at 45°C for 2 h. Purified OMC were obtained by removing cells by centrifugation at 12,000 g for 20 min and the supernatant was dialyzed overnight in 0.15 M NaCl. OMP was prepared as described by Zollinger, et al. [27] Protein concentration was determined by the method of Lowry, et al. [28].

Antibody screening by enzyme immunoassay (ELISA)

ELISA was performed as described by Harthug, et al. [29], with OMC or OMP at a protein concentration of 5 mg/mL as the coating antigen and a peroxidase–conjugated goat anti-mouse IgG (Sigma ,St.Louis,MO) as a detection system. For isotype determination,100 µl (5 µg/mL concentration), of OMP or OMC of the homologous strain B:4:P1.9 in phosphate buffered saline were dispensed into the wells of flat-bottom microtiter plates (Costar, Cambridge, MA) and allowed to adsorb for 18h at room temperature.

Dot-Blot

Dot-blot analyses were performed as described by Rosenquist, et al. [30]. Strain suspensions were made in phosphate buffered saline (PBS), pH 7.4, containing 0.02% sodium azide. The cells were heat inactivated at 56°C for 30 min, and the absorbance of the suspensions was adjusted to 1.0 at 650 nm using a spectrophotometer (model Spectronic 88). The bacterial cell suspension was stored at 4°C. For Dot-blot, 1 µl of meningococcal cell suspension was dotted onto 0.22 mm pore size Schleicher & Schuell (S.S) nitrocellulose strips. After drying, the strips were incubated for 1 h in a blotting buffer containing 2.5% bovine serum albumin (BSA) in PBS. The MAbs were pipetted directly into the blocking buffer according to the test for the standardization of the dilution of the MAbs used in the present study. The incubation in the presence of MAbs was carried out overnight at room temperature at 1:50,000 dilution. Next, the strips were separately washed 6 times with PBS. Antibody binding

Figure. 1. Immunoblots of (B:4:P1.9) OMC case strain of *Neisseria meningitidis* B reacted with monoclonal antibodies . **a)** class 1 (46 kD); **b)** class 3 (34kD); **c)** class 4 (32kD); **d)** class 5 (28kD)C14.F10Br2 (1:20,000); and **e)** class 5 (28 kD) 7F1.1B5Br3 (1:5,000); **f)** Molecular weight standards are indicated in kilodaltons. Solubilization for electrophoresis was done at 100 ºC
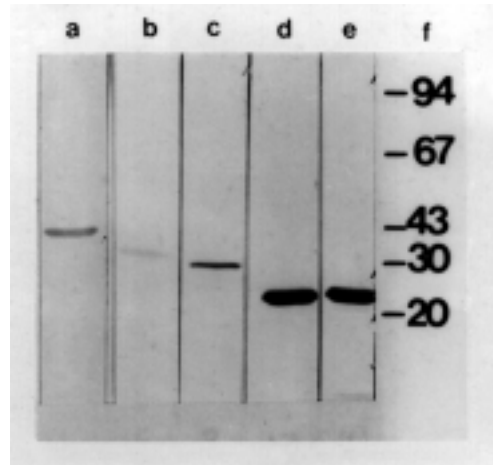
Figure 2. Dot plots of indirect immunofluorescence activated cell sorter analyses with whole cells (wc) of *N. meningitidis* B4:P1.9 (homologous strain). **a)** saline; **b)** unrelated IgG MAb; **c)** MAb 5F81A4 (class 1); **d)** MAb 5DC4-C8-C3 (class 4); **e)** MAb C14 F10 Br2 class (5.8); **f)** MAb 7F1 IB5 Br3 class (5.9). The monoclonals used in FACS analyses and the IgG-FITC labeled second antibody were used at 1:1000 dilution
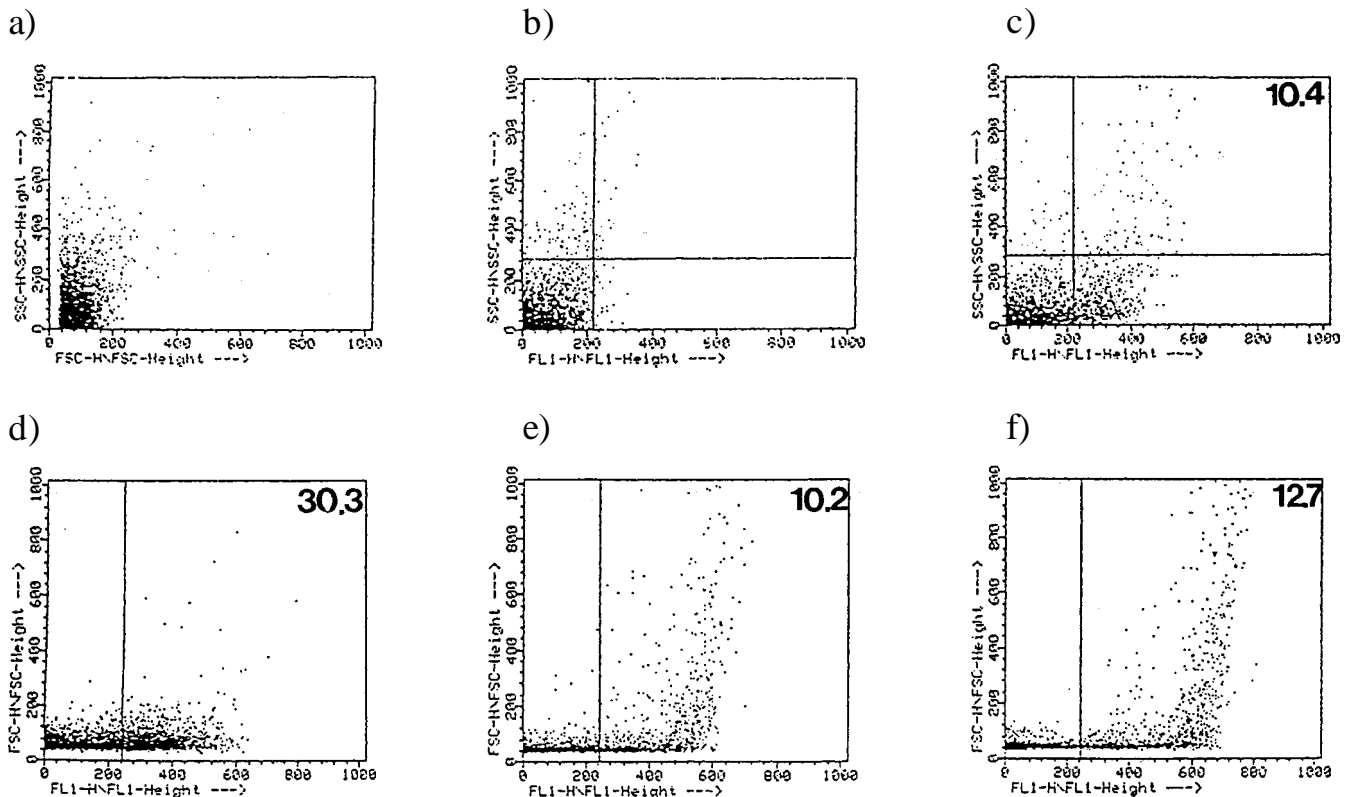
Table 1. Bactericidal titers of ascites fluids containing C14F10 (5.8) and 7F11B5 (5.9) monoclonal antibodies against *N. meningitidis* B:4:P1.9 (homologous strain)

Dilution	MAb (5.8)	MAb (5.9)
1:5000	88 %	96 %
1:10000	56 %	48 %

The reciprocal of the antibody dilution yielding 50% killing of meningococci was considered to be the bactericidal titer.

Table 2. ELISA reactivity using ascites and outer membrane complex (OMC) and outer membrane protein (OMP) antigens of *N.meningitidis* strain B:4:P1.9 (homologous strain)

Antigens	ELISA titer	
	MAb(5.8)	MAb(5.9)
OMC	1:128,000	1:64,000
OMP	1:256,000	1:128,000

Table 3. Frequency of class 5 expression in serogroups A, B, and C meningococci in Brazil during epidemics

Strains tested	Specificity of monoclonal antibodies used*						
	5.8	5.9	5.1	5.3	5.4	5.5	5c
Serogroup A (n=63))	NR	5.5%	NR	NR	NR	NR	47%
Serogroup B (n=136)	3.6%	8.0%	NR	NR	18%	22%	38%
Serogroup C (n=60)	NR	5.0%	81%	NR	35%	33%	NR

* C14F10Br2(5.8), 7F11B5Br3(5.9), D12(5.1), 3B4.C7(5.3), 1B61C7(5.4), 3DH9F5G8 (5.5) and 5F1F4.T3 (5c).
NR: no reactivity.

Table 4. Frequency of serotypes and subtypes expression in serogroups A, B, and C meningococcos in Brazil during epidemics

Strains tested	Monoclonal antibodies															
	Serotypes						Subtypes								
	4	14	19	21	2a	2c	1	2	3	7	9	15	16		
Serogroup A (n=63)	93%	NR	NR	NR	NR	NR	NR	NR	NR	NR	91%	NR	NR		
Serogroup B (n=136)	90%	1%	3%	1%	NR	NR	17%	1%	30%	12%	15%	41%	1%		
Serogroup C (n=60)	NR	NR	2%	NR	82%	2%	7%	60%	2%	NR	3%	NR	NR		

Serotypes: outer membrane antigens (class 2 or 3)
Subtypes: outer membrane antigens (class 1)
NR (no reactivity)

was detected after 2 h of incubation with a 1:1000 dilution peroxidase-conjugated of rabbit anti-mouse immunoglobulin (Dakopatts a/s, Denmark). The reaction was visualized by adding a freshly prepared solution containing 30% H_2O_2 and 18 mg/mL of 4-chloronaphthol in PBS.

This assay was used to determine the serotypes, as well as the class 5 epitopes, of Brazilian *Neisseria meningitidis* strains.

SDS-PAGE and immunoblot analysis

Whole cells of strain B:4:P1.9 were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels by the method of Laemmli [31]. The stacking gel contained 4% acrylamide, and the resolving gel contained 12% acrylamide. The samples were prepared by mixing 1 part of whole cells with 1.0 absorbance at 650 nm, with 1 part of sample buffer (62.5 mM Tris-hydrochloride, pH 6.8, 1% (v/v) glycerol, 2% (wt/v), 0.5% SDS (v/v), 2% mercaptoethanol, 0.5% (w/v) bromophenol blue and heated for 5 min at 100^0C or for 1 h at 37^0C. Samples (25 µl) were applied to each gel line. Electrophoresis was carried out at 50 V constant voltage until the bromophenol blue tracking dye entered the separating gel. A standard protein mixture (Pharmacia-LKB) was used for the determination of apparent molecular weight. After SDS-PAGE, proteins were transferred electrophoretically from the gel to nitrocellulose paper (S.S) by the methods of Towbin [32]. A constant potential of 5 V/cm was applied to the gel-nitrocellulose paper sandwich for 2 h in a semi-dry electroblotter, as specified by the manufacturer. The electroblotting buffer contained 25 mM Tris-hydrochloride, 192 mM glycine, and 20% (v/v) methanol, pH 8.3. Transferred proteins on the blot were stained with amido-black or detected by an enzyme immunoassay. To probe for bacterial antigens, the paper was soaked in 5% PBS-BSA solution for 2 h to block nonspecific protein binding sites, and then incubated overnight with monoclonal antibodies diluted in 1.25% PBS-BSA. The sheet was washed 4 times with PBS and incubated with

peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma), also diluted in 1.25% PBS-BSA. After 2 h incubation and 3 washes, the blots were soaked in a 30% H_2O_2 and 18 mg 4 chloronaphthol in PBS.

Bactericidal assay

The microbactericidal assay was done as described by Hoiby [33], with minor modifications. Two-fold dilutions of the ascitic fluid of MAb C14F10.Br2 (5.8) and 7F11B5Br3 (5.9) were tested with an inoculum of 70-80 cfu/well. A pool of normal sera obtained from a number of 4-week old rabbits was used as a source of complement, free of bactericidal antibody activity against group B. After 30 min at 37ºC, the material was plated onto TSB agar supplemented with 1% normal horse serum containing 2.5% vancomycin, colistin and nystatin (Sigma) to inhibit other flora. The bactericidal activity of MAbs 5.8 and 5.9 was compared with negative and positive controls.

Reactivity of the new MAbs with the surface of N. meningitidis B by flow cytometry

The ability of C14F10 (5.8) and 7F11B5 (5.9) to bind to the surface of pathogenic strains of *N. meningitidis* B was determined using flow cytometry with detection by indirect immunofluorescence assay. B:4:P1.9 *N. meningitidis* (whole cells) were washed and resuspended to an optical density of 1.0 at 650 nm in normal saline (NS) containing 1% (w/v) bovine serum albumin (Sigma). Bacteria were then mixed in 100 µl aliquots with MAb (ascitic fluid) diluted in 200 µl of NS/BSA and incubated at 37ºC for 30 min. Normal saline, or "nonsense" IgG MAbs, specific for unrelated proteins S, were employed as negative controls. Antibody-exposed bacteria were washed twice with NS/BSA and mixed with 200 µl of appropriately diluted FITC-labeled second antibody. The FITC-labeled probe was affinity-purified goat anti-mouse IgG. Bacteria were incubated with FITC-label antibodies for 30 min at 4ºC, washed twice with NS and resuspended in 1 mL of NS. As a control, bacteria were incubated in NS-1% BSA. All

samples were prepared in triplicate [34]. Positive control MAbs included meningococcal specific serotyping and subtyping. The cells were analyzed by flow cytometry with FACScan (Becton Dickinson, San Jose, CA, US). Each analysis included 5,000 events. One positive histogram gate was defined, allowing a maximum of 2% of the events of the control experiments to appear in the positive channels. Foward-scatter thresholds and gates were optimized to exclude cell debris and large bacterial aggregates from the analyses. MAb binding was expressed as the percentage of cells fluorescing more intensely than those stained with negative control and unrelated MAb. The monoclonals used in FACS analysis and the IgG-FITC labeled second antibody were used at 1:1000 dilution.

Results

Specificity of class (5.8) (5.9) MAbs

The specificity of the 2 new MAbs for class 5 epitopes is shown in the Immunoblot study (Figure 1). C14F10 (5.8) and 7F11B5 (5.9). MAb recognized a 28 kD protein in the outer membrane of *N. meningitidis*. To determine whether these MAbs were against known epitopes present in class 5, MAbs C14F10 (5.8) and 7F11B5 (5.9) were used together with existing anti-class 5 Mabs for reaction with a set of meningococcal reference strains. The results confirmed that both MAbs were different from those recognized by existing class 5 specific MAbs (P5.1, P5.3, and P5.4 and P5.5). The epitopes reactive with these MAbs were designated P5.8 and P5.9.

Bactericidal activity and ELISA

Table 1 shows bactericidal results, using the 2 new MAbs. The reciprocal of the antibody dilution producing 50% killing of the meningococci was considered to be the bactericidal titer. The 2 new monoclonal antibodies C14F10Br2 (5.8) and 7F11B5Br3 (5.9) reacted with OMP and OMC in ELISA, showing the binding of the 2 new MAbs was independent of the procedure used for obtaining the outer membrane antigens (Table 2). The 2 new MAbs, C14F10Br2 (IgG2a) and 7F11B5Br3 (IgG1), showed a strong antibody response to OMP and OMC in ELISA assay and have strong bactericidal activity.

Flow cytometry analysis

The ability of the C14F10 (5.8) and 7F11B5 (5.9) MAbs to bind to the (5.8) and (5.9) epitopes present on the surface of a pathogenic strain of *N. meningitidis* group B was also determined using FACScan analysis. One example of reactivity of MAbs specific for class 1, class 3, class 5.8 and 5.9 proteins with whole cells (wc) of a B:4:P1.9 strain can be observed in Figure 2.

Presence of class 5 epitopes

The whole cell dot blot assay was used to detect the percentage of strains with class 5 proteins expressing (5.1), (5.3), (5.4), (5.5), (5.8), (5.9) and (5.c) epitopes. Table 3 shows the prevalence of class 5 proteins recognized by the monoclonal antibodies on strains isolated in Brazil during epidemics.

Serotype and subtype patterns distribution of N. meningiditis in Brazil

The serogroups, serotypes, and subtypes of *N. meningitidis* B studied were: B:4:P1.15, B:4:P1.9, B:4:P1.7, B:4:P1.3, B:4:P1.14, B:4:P1.16, B:4:NT and B:NT:NT. The strains (C:2a:P1.3) and (A:4.21:P1.9) were the dominant serotypes and subtypes in *N. meningitidis* C and A, respectively (Table 4).

Discussion

Although the class 5 proteins of *N. meningitidis* induce antibodies that are strongly bactericidal against strains expressing the same protein, the occurrence of phase variation reduces the value of these antibodies in protection against group B disease and, thus, the value of their corresponding epitopes as vaccine components. A published paper reported [35] that the composition of the Brazilian meningococcal vaccine will

probably include a class 5 protein with the P5.5 epitope. However, a great heterogeneity of prevalent *N. meningitidis* B strains is found in Brazil, which was reflected in the bacterial populations under study as demonstrated in the present investigation.

Comparing the serogroup results obtained with a polyclonal antiserum from the National Reference Center for Meningitis, São Paulo, Brazil, with those obtained with the MAbs directed against serogroup-specific epitopes, discrepancies of only (2.5%) for serogroup A, and 3.5% for serogroup C, and 0% for serogroup B were observed. This shows the reliability of data on the serogrouping of *N. meningitidis* strains when Brazilian polyclonal antisera are used. These antisera are produced at the Immunology Division of the Adolfo Lutz Institute and distributed to various laboratories in Brazil for use in counterimmunoelectrophoresis and agglutination assays [24].

The vaccine for *N. meningitidis* B, to be prepared in Brazil should contain the (5.5) antigen and (5c), because we found the expression of 5c to be highly significant. The new MAbs showed that C14F10 (5.8) reacted with 3.6% of the serogroup B strains investigated, while MAb 7F11B5 (5.9) reacted with 8.8% of serogroup A, 5% of serogroup B, and 5% of serogroup C strains of *N. meningitidis* isolated during epidemics in Brazil.

Studies on the 5c protein suggest that future OMV vaccines should be prepared from strains expressing the 5c protein, but this protein alone will not provide broad protection [22]. On the other hand, this protein is probably not a true class 5 protein [36], although Achtman, et al., considered it a class 5 variant [37]. Whereas class 5 proteins are heat modifiable and antigenically diverse, and their expression is controlled by multiple repeats of the nucleotide sequence CTCTT, the 5c protein is not heat modifiable, is antigenically conserved, and is not controlled by the CTCTT sequence. The level of 5c expression is variable, a fact that led Achtman, et al. [37], to designate the strains as 5c and 5C according to the quantity of protein expressed. The 5c protein is expressed in about 20% of serogroup B disease isolates [38].

Our studies using *N. meningitidis* B from the year of 1992, showed that class 5c had a prevalence of 30% in local Brazilian strains. The 5c protein is a possible vaccine candidate because it induces bactericidal antibodies. These antibodies may also block tissue invasion. The protein appears to be involved in adherence to, and invasion of, endothelial and epithelial cells. In an *in vivo* model, it was shown that strains lacking the 5c protein were largely unable to invade cells, and that antibodies to 5c could interfere with attachment and penetration [39]. Rosenqvist, et al. [22], found that antibodies to 5C were strongly bactericidal only for strains expressing large amounts of 5C. Identical results were obtained with 2 human monoclonal antibodies to the 5C protein [40]. Elevated 5c antibody levels were also seen in convalescent sera, but not in acute sera from meningococcal disease patients. Thus, the protein is antigenically conserved and immunogenic in humans, inducing potentially protective antibodies [22].

Considering the high incidence of meningococcal disease due to certain group B strains in Brazil, the studies of surface structures of class 5 proteins are important. Groups in different Brazilian laboratories have emphasized the importance of class 5 proteins [34, 41, 42] in Brazil. However, no analysis of the prevalence of class 5 types expressed in serogroup B strains in Brazil has been performed, despite the importance of such an analysis for the identification of prevalent class 5 proteins in our strains. The present study shows, for the first time, the importance of Opc (5c) as one of the components of the vaccine to be produced in Brazil. Thus, besides the use of the 5.5 antigen, a broader range of class 5 antigens must be included in a vaccine.

As reported in the literature [43], it is important to establish a broad set of antigens characteristic of the prevalent meningococcal species found in each epidemic region in order to develop truly effective vaccines and immunoprophylactic programs.

Acknowledgements

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Pneumocystis carinii Pneumonia, Pulmonary Tuberculosis and Visceral Leishmaniasis in an Adult HIV Negative Patient

Antonio Carlos Toledo Jr. and Márcio Rodrigues de Castro

Eduardo Menezes Hospital, Hospitalar Foundation of Minas Gerais, MG, Brazil

This is a case report of a 29 year old male with pneumocystis pneumonia and tuberculosis, and who was initially suspected of having HIV infection, based on risk factor analyses, but was subsequently shown to be HIV negative. The patient arrived at the hospital with fever, cough, weight loss, loss of appetite, pallor, and arthralgia. In addition, he was jaundiced and had cervical lymphadenopathy and mild heptosplenomegaly. He had interstitial infiltrates of the lung, sputum smears positive for *Mycobacterium tuberculosis* and *Pneumocystis carinii*, and stool tests were positive for *Strongyloides stercoralis* and *Schistosoma mansoni*. He was diagnosed as having AIDS, and was treated for tuberculosis, pneumocystosis, and strongyloidiasis with a good response. The patient did not receive anti-retroviral therapy, pending outcome of the HIV tests. A month later, he was re-examined and found to have worsening hepatosplenomegaly, pancytopenia, fever, and continued weight loss. At this time, it was determined that his HIV ELISA antibody tests were negative. A bone marrow aspirate was done and revealed amastigotes of leishmania, and a bone marrow culture was positive for *Leishmania* species. He was treated with pentavalent antimony, 20 mg daily for 20 days, with complete remission of symptoms and weight gain. This case demonstrates that immunosuppression from leishmaniasis and tuberculosis may lead to pneumocystosis, and be misdiagnosed as HIV infection. The occurrence of opportunistic infections in severely ill patients without HIV must always be considered and alternate causes of immunosuppression sought.
Key Words: *Pneumocystis carinii*, visceral leishmaniasis, tuberculosis, HIV diagnosis, opportunistic infections.

We describe a case of *P. carinii* pneumonia as a complication of visceral leishmaniasis in an HIV negative patient. Visceral leishmaniasis (VL) is a chronic infectious disease characterized by an important immunological dysfunction that predisposes to other infections, mainly involving the pulmonary and gastrointestinal systems. In Brazil, bacterial pneumonia and tuberculosis are frequent opportunistic infections in this disease. *P. carinii* is a ubiquitous organism that commonly causes disease in immunodeficient patients such as in cancer, HIV/AIDS, leukemia, lymphoma, and transplant recipients [1]. Although there is no description of *P. carinii* pneumonia as an opportunistic infection of VL in the medical literature, it is plausible that immunodysfunction induced by Leishmania could be responsible for this patient's illness. If so, this would be the first description of an association of VL and *P. carinii* pneumonia.

Case report

A 29 year old male patient, was admitted to Hospital Eduardo de Menezes (Infectious Diseases Hospital of Minas Gerais State, Brazil) on October 16, 1995. He complained of fever, asthenia, weight loss (>10 kg), loss of appetite, generalized arthralgia and a productive cough. Past history indicated 2 blood transfusions and