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Serological Specificities of Murine Hybridoma Monoclonal Antibodies against *Neisseria meningitidis* Serogroups B, C, Y, and W135 and Evaluation of Their Usefulness as Serogrouping Reagents by Indirect Whole-Cell Enzyme-Linked Immunosorbent Assay

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Murine hybridoma monoclonal antibodies (MAbs) were produced against the capsular antigens of serogroups B, C, Y, and W135 meningococci. Each serogroup-specific MAb reacted with the extracted capsular polysaccharide from its homologous serogroup only and did not react with capsules from the other three serogroups. The application of these MAbs for serogroup identification of meningococci was demonstrated by their abilities to correctly identify 183 clinical isolates of 185 meningococci recovered from individual invasive meningococcal disease (IMD) patients during routine surveillance in 2002. The remaining two meningococci were identified by PCR grouping as C in one case and Y in another, but neither isolate was positive by bacterial agglutination using rabbit antisera or by enzyme-linked immunosorbent assay using MAbs. The specificities of the anti-Y and anti-W135 MAbs were further assessed by tests with 37 serogroup W135 and 106 serogroup Y meningococci recovered from IMD cases during 1999 to 2001 and 2003. All 143 meningococci except one serogroup Y isolate were correctly identified by positive reactions with the corresponding MAbs that identified their homologous serogroups. The single serogroup Y isolate was received as nonagglutinable and tested as negative with both rabbit anti-Y antiserum and anti-Y MAb but was positive for the serogroup Y-specific *siaD* gene. The advantage of using MAbs for serogrouping of meningococci is discussed.

Neisseria meningitidis remains a significant human pathogen and a leading cause of meningitis and septicemia in adults as well as in children. Besides causing a significant case fatality rate of about 9 to 12% (27), complications and postinfectious sequelae are also common (12, 14). Of the 13 known serogroups, classified based on serological specificities of their capsular antigens (30), most (>90%) diseases are caused by organisms belonging to serogroups A, B, C, Y, and W135 (21, 24). In Canada as well as in the United States, diseases caused by serogroup A organisms are no longer endemic, and most endemic as well as epidemic diseases are now caused by organisms belonging to serogroups B, C, Y, and W135 (26, 28).

Serogrouping of *N. meningitidis* (or meningococci) is important because the disease caused by some serogroups can be prevented by active immunization. Therefore, in outbreak situations as well as in routine surveillance of this disease, results of serogroup determination may lead to decisions on the public health intervention measures to be taken. Serogroup determination may also help in understanding the changing epidemiology of meningococcal disease (26, 28). Since serogroup classification is based on unique antigens present on the capsules of meningococci, traditionally serogrouping is done by detection of these antigens with specific antisera using the simple bacterial agglutination test. Most serogrouping antisera are produced in rabbits, but antisera towards carbohydrate antigens are usually of low titer, especially in the case of antisera to serogroup B meningococci, because of the molecular mimicry between the serogroup B capsule and the tissue antigen of neuron cell adhesion molecule (20). Therefore, antisera are usually used without cross-absorption with related serogroups of meningococci to remove antibodies to the common outer membrane protein antigens, and specificity of such polyclonal antisera remains an issue. For example, in a study to compare commercial serogrouping antisera for the identification of serogroup antigens of *N. meningitidis*, it was found that such reagents had good sensitivities and specificities for detection of the more common serogroups A, B, and C but were not satisfactory for determination of the uncommon serogroups, including Y and W135 (29).

Besides bacterial agglutination, other methods have been proposed for serogrouping of meningococci, and these include an antiserum agar method (2, 3, 9, 10), coagglutination with serogroup-specific antibodies coated onto protein A-rich staphylococci (32), latex agglutination (16), membrane-based dot immunoassay (23), and enzyme-linked immunosorbent assay (ELISA) (31). Also, monoclonal antibodies (MAbs) to the serogroup-specific antigens of *N. meningitidis* serogroup A, B, and C bacteria have also been described (5, 13, 22). Detection of the serogrouping antigens by antibodies may sometimes give negative results because of down-regulation of the capsule synthesis genes, and this is especially common among isolates recovered from carriers (8). Therefore, a number of molecular methods have also been proposed for determination of the serogroup nature of meningococci. Most of these molecular methods are PCR-based assays designed to detect either the

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serogroup-specific *siaD* gene in serogroups B, C, Y, and W135 (6, 7) or the serogroup A capsule-specific *myn* gene (11), or specific regions towards the 5′ end of the *ctrA* genes for serogrous 29E, X, and Z (4). Real-time PCR (19, 25) and nucleotide sequence analysis (17) have been proposed for identification of serogroup Y and W135 meningococci.

In this paper, we describe the serological specificities of murine hybridoma MAbs against the capsular antigens of four common serogroups of meningococci in North America (B, C, Y, and W135), and we report their usefulness as serogrouping reagents.

MATERIALS AND METHODS

***N. meningitidis* strains and biochemical identification.** Isolates of *N. meningitidis* from invasive meningococcal disease (IMD) cases, defined as a patient presenting with symptoms compatible with meningococcal disease such as meningitis, septicemia, petechiae-purpura, and fever, were selected for this study. They were submitted to Health Canada's National Microbiology Laboratory by provincial and territorial public health laboratories across Canada for the national surveillance of IMD (28). Their identities were confirmed by standard biochemical tests. A total of 185 isolates collected from individual IMD cases in 2002 were included for this study. These 185 isolates included 77 serogroup B, 66 serogroup C, 29 serogroup Y, 6 serogroup W135, and 7 isolates that were nonserogroupable. Because of the small number of serogroup Y and W135 isolates collected in 1 year, additional serogroup Y (a total of 106 isolates) and serogroup W135 (a total of 37 isolates) meningococci collected within the years 1999 to 2001 and 2003 were also used in this study.

Production of polyclonal rabbit antisera and murine hybridoma MAbs to serogroup antigens of *N. meningitidis*. Rabbit antisera to the capsular polysaccharide antigens of serogroups A, B, C, W135, X, Y, Z, and 29E were produced according to the method described by Ashton et al. (2). Albino rabbits were immunized by intravenous injections of a viable suspension of the bacteria adjusted to a cell density equivalent to a turbidity of a McFarland number 3 standard. The dosage and schedule of the immunizations involved 3 injections per week given on alternate days with 0.2, 0.2, and 0.3 ml for week 1, followed by three 0.5-ml injections in week 2 and 1.0, 1.0, and 1.5 ml for week 3.

Murine MAbs to the capsular antigens of serogroups B, C, W135, and Y were produced by the standard hybridoma method of fusing immune splenocytes from BALB/c mice with myeloma cells, as described by Kohler and Milstein (15) and Mandrell and Zollinger (18). Mice were immunized with live bacterial whole cells suspended in sterile phosphate-buffered saline [PBS] to give a cell density (optical density [OD] at 600 nm) of about 0.25, given by both subcutaneous (0.05 ml) and intravenous (0.05 ml) routes twice either 1 or 2 months apart. Isotypes of the MAbs were determined with the mouse MAb isotyping kit (Roche Diagnostics, Laval, Quebec, Canada).

Bacterial agglutination. The bacterial agglutination test to determine the serogroup identity of meningococci was done as described by Ashton et al. (2).

ELISA. Serogrouping by MAbs was done by an indirect whole-cell ELISA, which was a modification of the method of Abdillahi and Poolman (1) described for typing of *N. meningitidis*. Briefly, to prepare the ELISA antigens for testing, an overnight growth of *N. meningitidis* was resuspended in pH 7.4 sterile PBS and the bacteria were inactivated in a 56°C water bath for 60 min. Inactivated bacterial cells were stored at 4°C until ready for testing. To coat ELISA plates, inactivated meningococci were diluted in sterile PBS and adjusted to give an absorbance of about 0.1 at a 620-nm wavelength. For antigen coating, 100 μl of the diluted cell suspension was added in duplicate to wells of a Nunc Maxisorp immunoplate (Nalge Nunc International, Rochester, N.Y.), and the bacteria were allowed to dry onto the wells at 37°C overnight. After three washes with 0.85% saline and 0.05% Tween 20 (saline-Tween), wells were blocked with 2% bovine serum albumin in PBS (BSA-PBS) at 37°C for 90 min. Suitable dilutions (determined by prior titrations) of murine MAbs to serogroup B, C, W135, and Y meningococci were added to both antigen-coated and no-antigen control wells for incubation at 4°C overnight. Meningococci of known serogroups were used as positive controls. After four washes with saline-Tween, MAbs bound to the plate were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) $F(ab')_2$ fragment-specific antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted 1:5,000 in 2% BSA-PBS for incubation at room temperature for 4 h. After a final four washes with saline-Tween, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate (Roche Diagnostics) was added for color development at room temperature. ELISA readings were taken at 405 nm using a Dynatech ELISA reader against a row of blank wells of ABTS substrate (without antigens and without primary and secondary antibodies). All reagents were added in a volume of 100 μl per microtiter well with the exception of the blocking and washing steps, where 300 μl per well was used. Although the amounts of MAbs bound to homologous serogroups of meningococci may be highly variable, possibly due to variable expression of capsular polysaccharides in strains, the background signals due to nonspecific binding of MAbs to no-antigen control wells as well as to heterologous serogroups of meningococci were typically low (ELISA OD of less than 0.05 in most cases; for details, see results presented below in Tables 2, 3, and 4). All tests were done in duplicate, and the two OD readings were always within 10% variation from each other, indicating good intra-assay reproducibility. To determine interassay reproducibility, the same positive control was tested on five consecutive assay days and the coefficient of variation was typically within 15%. A strain was regarded as positive with a MAb when the ELISA OD obtained was at least three times above the background of either the no-antigen control or the negative readings obtained with the other three MAbs.

When testing MAbs for their reactions with the extracted capsules, purified capsular polysaccharides from serogroups C, Y, and W135 were purchased from the National Institute of Biological Standards and Control (Potters Bar, Hertfordshire, United Kingdom), while capsule polysaccharides from serogroup B were extracted and purified by a method similar to that described by Nato et al.

TABLE 1. Antigenic specificities of antimeningococcal MAbs against extracted capsular polysaccharides by indirect ELISA

MAb and dilution	Mean[a] ELISA OD against polysaccharides extracted from serogroup:			
	B	C	Y	W135
Anti-B, 2-2-B				
1:200	**2.371**[b]	0.003	0.002	0.003
1:500	**1.244**	0.001	0.000	0.001
Anti-C, 4-2-C				
1:500	0.005	**3.919**	0.001	0.002
1:5,000	0.000	**3.884**	0.002	0.001
Anti-Y, 5-1-Y				
1:500	0.000	0.002	**3.041**	0.002
1:5,000	0.001	0.001	**0.344**	0.001
Anti-W135, 6G9-7				
1:500	0.002	0.002	0.001	**3.154**
1:5,000	0.002	0.002	0.000	**0.557**

[a] Mean of duplicate determinations at 30 min.
[b] Bold values indicate positive results for binding of serogroup-specific capsular polysaccharide.

TABLE 2. Reactions of anti-B, anti-C, anti-Y, and anti-W135 MAbs against 13 known serogroups of meningococci

Serogroup (strain)	Mean[a] ELISA OD with MAb			
	Anti-B	Anti-C	Anti-Y	Anti-W135
A (2E)	0.012	0.011	0.028	0.014
B (99M)	**0.846**[b]	0.015	0.036	0.019
C (60E)	0.022	**3.410**	0.026	0.016
D (1613)	0.044	0.040	0.215	0.117
29E (521)	0.021	0.021	0.152	0.066
H (1890)	0.018	0.014	0.150	0.066
I (1486)	0.022	0.020	0.152	0.073
K (7811)	0.025	0.024	0.119	0.060
L (76189)	0.025	0.021	0.176	0.110
W135 (III)	0.014	0.011	0.040	**2.973**
X (Slaterus X)	0.012	0.012	0.030	0.017
Y (Slaterus Y)	0.020	0.021	**1.987**	0.058
Z (Slaterus Z)	0.026	0.025	0.066	0.037
No-antigen control	0.001	0.004	0.000	0.002

[a] Mean of duplicate determinations.
[b] Values in bold indicate positive results for binding of serogroup-specific capsular polysaccharide.

TABLE 3. Comparison of polyclonal versus monoclonal antibodies for serogroup identification of serogroup Y and W135 meningococci

Rabbit antisera[a]	No. of isolates	MAb[b]	Positive reaction ODs			Negative reaction ODs		
			Mean	SD	Range	Mean	SD	Range
B	77	B	1.268	0.385	0.326–2.043	0.026	0.021	0–0.123
C	66	C	0.735	0.834	0.875–3.5	0.024	0.028	0–0.095
Y	29	Y	1.782	0.560	0.617–2.615	0.014	0.019	0–0.076
W135	6	W135	1.803	0.929	0.722–2.912	0.024	0.030	0–0.096
Nongroupable	1[c]	B	1.581	NA[e]	NA	0.057	NA	NA
Nongroupable	4[c]	C	2.250	0.826	1.236–3.113	0.040	0.024	0.003–0.088
Nongroupable	2[d]	Negative	NA	NA	NA	0.046	NA	0.014–0.076

[a] In-house-produced antisera and serogroup determined by bacterial agglutination method.
[b] Serogroup determined by indirect whole-cell ELISA.
[c] Grouping confirmed by PCR detection of serogroup-specific *siaD* genes.
[d] Identified by PCR detection of serogroup-specific *siaD* genes as C (one isolate) and Y (one isolate).
[e] NA, not applicable.

(22). Capsule polysaccharide antigens were coated at a concentration of 10 μg per ml in PBS at 4°C overnight. The rest of the procedure was similar to the indirect whole-cell ELISA method described above.

Grouping by PCR detection of serogroup-specific *siaD* gene. Grouping of meningococci by PCR amplification and hybridization (PCR-ELISA) to detect the *siaD* genes specific to serogroups B, C, Y, and W135 were done essentially as described by Borrow et al. (6, 7).

RESULTS

Specificities of murine hybridoma MAbs. Four MAbs were selected for this study. They were 2-2-B (IgM/κ) for serogroup B, 4-2-C (IgG3/κ) for serogroup C, 5-2-Y (IgM/κ) for serogroup Y, and 7-1-W (IgG3/κ) for serogroup W135. All four MAbs were found to react with extracted capsules from the homologous serogroup but did not react with related polysaccharides from the heterologous serogroups (Table 1). When each of the four MAbs was tested against all 13 known serogroups of meningococci by indirect whole-cell ELISA, the anti-B and anti-C MAbs were found to be highly specific and showed strong reactions with meningococci of the homologous serogroups only, without any evidence of cross-reactions with cells of the heterologous serogroups. However, the anti-Y (5-1-Y) and anti-W135 (7-1-W) MAbs showed traces of cross-reactions with cells of other serogroups besides reacting strongly with their homologous serogroups. The anti-Y MAb was found to give weak reactions with cells of serogroup D, 29E, H, I, K, and L meningococci, while the anti-W135 MAb was found to react weakly with cells of serogroups D and L. In both cases, reactions of the MAbs with cells of their homologous serogroups gave ELISA ODs much higher than ODs observed in the cross-reactions (Table 2).

Evaluation of MAbs as reagents for serogrouping of meningococci. A total of 178 strains out of a total of 185 meningococci collected from IMD cases in 2002 gave concordant results when their serogroups were determined by using two different kinds of serogrouping antibodies and two different serological methods (rabbit polyclonal antisera used in a slide agglutination test versus mouse MAbs used in an ELISA) (Table 3). Five isolates that were nongroupable (because they were either nonagglutinable or polyagglutinable) with rabbit antisera were identified by MAbs to be serogroup B (one isolate) and serogroup C (four isolates). Two isolates were found to be negative for the presence of serogroup antigens regardless of whether rabbit antisera or mouse MAbs were used. These two isolates were identified by PCR grouping as C (one isolate) and Y (one isolate).

The reactions of 37 serogroup W135 and 106 serogroup Y isolates with the anti-Y and anti-W135 MAbs are summarized in Table 4. All 37 serogroup W135 isolates gave clear-cut strong reactions with the anti-W135 MAb (mean ELISA OD, 1.838; standard deviation, [SD], 0.835; range, 0.939 to 3.916) and did not react with the anti-Y MAb (mean ELISA OD, 0.039; SD, 0.02; range, 0.008 to 0.089). A total of 105 out of 106 serogroup Y isolates were detected by the anti-Y MAb (mean ELISA OD, 0.711; SD, 0.314; range, 0.212 to 1.848). Their reactions with the anti-W135 MAbs gave a mean ELISA OD of 0.025 with a range of 0.008 to 0.054 and an SD of 0.009. The single serogroup Y isolate that did not react with the anti-Y MAb was submitted to our laboratory as a nonagglutinable isolate, and it was identified as serogroup Y by *siaD* PCR-ELISA.

DISCUSSION

The specificities of four murine hybridoma MAbs to serogroup B, C, Y, and W135 meningococci were confirmed by their reactions towards the extracted capsular antigens from

TABLE 4. Specificity testing of anti-Y and anti-W135 MAbs against *N. meningitidis* serogroup Y and W135 isolates obtained from Canadian IMD cases in 2003 and 1999 to 2001

Serogroup[a]	No. of strains	No. identified by MAbs	No. negative with MAbs	Positive reaction ODs			Negative reaction ODs		
				Mean	SD	Range	Mean	SD	Range
W135	37	37	0	1.838	0.835	0.939–3.916	0.039	0.020	0.008–0.089
Y	106	105	1[b]	0.711	0.314	0.212–1.848	0.025	0.009	0.008–0.054

[a] Serogroups identified by bacterial agglutination with rabbit antisera (2).
[b] The single isolate was identified as group Y by PCR detection of the serogroup Y-specific *siaD* gene.

homologous serogroups of meningococci without any traces of cross-reactions with the polysaccharides from the other three serogroups. This was confirmed with bacterial whole cells as antigens in an indirect whole-cell ELISA (Table 2). The small amount of reactivity found for the anti-Y and anti-W135 MAbs with serogroup D, 29E, H, I, K, and L meningococci cannot be readily explained from our current knowledge about the capsular and/or other surface structures of these serogroups of meningococci. Nevertheless, these minor cross-reactions should not pose any problem in routine use of the anti-Y and anti-W135 MAbs, since serogroup D, H, I, K, and L meningococci are rare in North America and their reactions with the anti-Y and anti-W135 MAbs gave much lower ELISA ODs (in the range of 0.110 to 0.215) compared to the reactions of serogroup Y meningococci reacting with anti-Y MAb (ELISA OD, 1.987) and serogroup W135 meningococci reacting with anti-W135 MAb (ELISA OD, 2.973).

With regards to serogroup 29E meningococci, a specific MAb is also available in our laboratory, and preliminary testing has shown that this antibody is specific for its homologous serogroup (W. D. Zollinger, unpublished data). Besides reacting with the serogroup B meningococci, the anti-serogroup B MAb 2-2-B also reacted with K1-positive *Escherichia coli* strains, but it did not react with K1-negative *E. coli* isolates (A. Henderson and R. S. W. Tsang, unpublished data).

Serological cross-reaction between serogroups Y and W135 has been described, and it is usually ascribed to the antigenic similarities of their capsular polysaccharides (8, 9). For example, it has been observed with the antiserum agar method that serogroup Y isolates produce immunoprecipitation lines with not only anti-Y antiserum but also with anti-W135 antiserum (9, 10). We have also observed isolates showing polyagglutinations in both anti-Y and anti-W135 meningococci (National Microbiology Laboratory, unpublished observations). We therefore examined the reactions of anti-Y and anti-W135 MAbs against most of the serogroup Y and W135 meningococci collected in our laboratory from IMD cases over the last few years. Testing with 106 serogroup Y isolates and 37 serogroup W135 bacteria confirmed the specificities of these two MAbs (Table 4).

The routine application of MAbs in serogroup determinations was shown by the results presented in Table 3. Five isolates that were either nonagglutinable or polyagglutinable in the bacterial agglutination test with rabbit serogrouping antisera were identified by MAbs, thus showing an advantage of the ELISA method over the bacterial agglutination test. ELISA or dot blot immunoassay is routinely used in meningococcal reference laboratories for identification of serotype and serosubtype antigens of meningococci (1). Therefore, serogrouping with MAbs using the ELISA method does not require a methodology that is not already being used. A further advantage of the ELISA method over the bacterial agglutination test is the fact that isolates that tend to autoagglutinate can still be grouped, since the binding of MAbs to the meningococcal cells is detected by an enzyme label instead of relying on the bacterial agglutination of the antigen-antibody binding reaction. Finally, detection of the presence of the capsular antigens confirmed the expression of the antigens, while detection of a specific *siaD* gene does not allow one to predict whether capsule will be expressed or not.

Besides using the MAbs in a whole-cell ELISA, we have also produced high titers of these antibodies under tissue culture conditions using Integra flasks (Integra Biosciences Inc., Zurich, Switzerland), and these high-titer antibodies (in the range of over 1:100,000 by ELISA detection) allow meningococci to be agglutinated in a specific fashion, just like when using polyclonal rabbit hyperimmune antiserum (D. Law, A. Henderson, and R. S. W. Tsang, unpublished observation). We therefore propose that MAbs have advantages over polyclonal antisera for serogroup identification of meningococcal isolates recovered from IMD cases. Besides being highly specific, grouping with MAbs by the ELISA method is objective and does not depend on isolates that will not provide a smooth nonautoagglutinating suspension for the test to be valid. In our experience, fresh meningococcal isolates recovered from IMD cases are almost invariably encapsulated and can therefore be grouped by the proposed method and monoclonal reagents.

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