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Bactericidal Monoclonal Antibodies That Define Unique Meningococcal B Polysaccharide Epitopes That Do Not Cross-React with Human Polysialic Acid[1]

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The poor immunogenicity of the *Neisseria meningitidis* group B polysaccharide capsule, a homopolymer of $\alpha(2\rightarrow8)$ sialic acid, has been attributed to immunologic tolerance induced by prenatal exposure to host polysialyated glycoproteins. Substitution of *N*-propionyl (*N*-Pr) for *N*-acetyl groups on the meningococcal B polysaccharide, and conjugation of the resulting polysaccharide to a protein carrier, have been reported to yield a conjugate vaccine that elicits protective Abs with minimal autoantibody activity. To characterize the protective epitopes on the derivatized polysaccharide, we isolated 30 anti-*N*-Pr meningococcal B polysaccharide mAbs. These Abs were heterogeneous with respect to complement-mediated bactericidal activity, fine antigenic specificity, and autoantibody activity as defined by binding to the neuroblastoma cell line, CHP-134, which expresses long-chain $\alpha(2\rightarrow8)$-linked polysialic acid. Eighteen of the Abs could activate complement-mediated bacteriolysis. Seven of these 18 Abs cross-reacted with *N*-acetyl meningococcal B polysaccharide by ELISA and had strong autoantibody activity. Thus, *N*-Pr meningococcal B polysaccharide conjugate vaccine has the potential to elicit autoantibodies. However, 7 of the 18 bactericidal mAbs had no detectable autoantibody activity. These Abs may be useful for the identification of molecular mimetics capable of eliciting protective Abs specific to the bacteria, without the risk of evoking autoimmune disease. ***The Journal of Immunology*****, 1998, 160: 5028–5036.**

With the control of *Haemophilus influenzae* type b disease by universal infant vaccination, *Neisseria meningitidis* has emerged as the most common cause of bacterial meningitis and an important cause of sepsis in the United States and many other industrialized countries (1–3). Pathogenic meningococci can be subdivided into serogroups based on immunologically and chemically distinctive polysaccharide capsules. Five major serogroups, designated A, B, C, Y, and W135, account for nearly all disease-producing isolates. Serogroup A isolates are the major cause of disease in tropical countries, whereas serogroups B and C strains account for the vast majority of invasive meningococcal isolates in industrialized countries (1–3).

Polysaccharide vaccines are available for prevention of disease caused by serogroup A, C, Y, and W135 strains. These vaccines are used infrequently because plain polysaccharide vaccines tend to be poor immunogens in infants and young children, the age groups with the highest incidences of meningococcal disease. Efforts are underway to develop more effective meningococcal A and C vaccines by conjugating the polysaccharides to protein carriers using approaches that proved highly effective for *Haemophilus* type b conjugate vaccines (4–6). However, no vaccine is currently available for prevention of disease caused by serogroup B strains, which account for 50% or more of invasive isolates in Europe and North America (2, 3). Further, as described below, the conventional conjugate vaccine approach has not proven promising for prevention of disease caused by meningococcal serogroup B strains.

The group B polysaccharide capsule is composed of a homolinear polymer of $\alpha(2\rightarrow8)$ *N*-acetyl (*N*-Ac)[3] neuraminic acid (polysialic acid). This capsule is an important virulence determinant: for example, mutants deficient in capsular expression are serum sensitive and nonpathogenic. Evidence also indicates that serum Ab to the group B polysaccharide confers protection against disease by activating complement-mediated bacteriolysis and/or opsonization (4, 7). Efforts to employ the meningococcal B capsular polysaccharide as a vaccine component have been hampered by its very poor immunogenicity, even when conjugated to a carrier protein (8). The poor immunogenicity is attributed to immunologic tolerance induced by fetal exposure to cross-reactive polysialylated glycoproteins, such as the neural cell adhesion molecule, expressed in a variety of host tissues (9, 10). Jennings, Roy, and Gamian (11); Jennings, Gamian, and Ashton (12); and Jennings (13) have pursued an innovative strategy for overcoming immunologic tolerance to this polysaccharide by substitution of *N*-propionyl (*N*-Pr) for *N*-Ac groups, and conjugation of the resulting *N*-Pr meningococcal B polysaccharide to a protein carrier. The resulting conjugate vaccine is reported to be highly immunogenic in experimental animals, including nonhuman primates (14), eliciting IgG Abs that activate complement-mediated bacteriolysis and

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[3] Abbreviations used in this paper: *N*-Ac, *N*-acetyl; *N*-Pr, *N*-propionyl; Dp, degree of polymerization; NCAM, neuronal cell adhesion molecule.

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passively protect experimental animals challenged with *N. meningitidis* group B. These Abs also appear to have less autoantibody activity than Abs raised to the native meningococcal B polysaccharide.

One potential concern about the use of the Jennings *N*-Pr meningococcal B conjugate vaccine is that a subset of the Abs elicited by this vaccine have anti-host Ab activity (see, for example, Fusco et al. (14) and Häyrinen et al. (15)). Although there is no direct evidence that such autoantibodies are deleterious to the host, it is difficult to eliminate this possibility. For example, Abs capable of binding to host polysialic acid could evoke autoimmune inflammatory disease, or could cross the placenta and adversely affect neurologic development in the fetus (16, 17).

The ideal meningococcal B vaccine should elicit protective Abs but pose a minimal risk of evoking autoantibodies. Given that it is both unlikely and difficult to prove that meningococcal B polysaccharide-based vaccines do not elicit autoantibodies, alternatives must be considered. One approach is to use noncapsular Ags (reviewed in Ref. 4). However, because of strain heterogeneity, and temporal and geographic shifts in the predominant strains causing disease, this approach requires the use of Ags from multiple meningococcal B strains. Another approach would be to use molecular mimetics, such as peptides, of meningococcal B polysaccharide epitopes that are unique to the pathogen. This approach has been used successfully by Westerink et al. to induce Abs to meningococcal C polysaccharide ($\alpha(2\rightarrow9)$ *N*-Ac neuraminic acid (18)).

The purpose of the present study was to prepare a panel of murine mAbs to *N*-Pr meningococcal B polysaccharide, with the goal of identifying Abs that react with epitopes that are specific for meningococcal B organisms and that show no cross-reactivity or minimal cross-reactivity with host polysialic acid. Such Abs could be useful for identifying molecular mimetics for inclusion in a meningococcal B vaccine.

Materials and Methods

Preparation of conjugate vaccine

N-Ac meningococcal B polysaccharide was purified from the supernatant of broth cultures of *Neisseria meningitidis* group B (strain B11), as described by Bartoloni et al. (19). For preparation of the derivatized polysaccharide, the *N*-Ac groups of *N*-Ac meningococcal B polysaccharide were removed by heating a solution of the polysaccharide (13.3–16.6 mg/ml) in 2 M NaOH to 110°C for 6 h in the presence of sodium borohydride (1 mg/ml), as described by Jennings, Roy, and Michon (20). The deacetylated polysaccharide was exhaustively dialyzed in saturated sodium bicarbonate buffer, then stirred with an excess of propionic anhydride for 12 h at ambient temperature. The solution was exhaustively dialyzed in water and the *N*-propionylated meningococcal B polysaccharide was recovered by lyophilization.

For preparation of the conjugate vaccine, the *N*-Pr meningococcal B polysaccharide was partially hydrolyzed in 10 mM sodium acetate, pH 5.5, at 50°C for 2 h. The resulting mixture of oligosaccharides was fractionated on a Q-Sepharose FF column (Pharmacia, Uppsala, Sweden) (approximately 10–15 mg of sialic acid per ml of gel) by using a stepwise gradient elution consisting of low-salt (100 mM sodium chloride) and high-salt (500 mM sodium chloride) solutions in 5 mM sodium acetate buffer, pH 6.5. As determined by analytical chromatography on a MonoQ column (Pharmacia) small oligosaccharides with a degree of polymerization (Dp) ranging from 2 to 6 were eluted with low-salt buffer, and intermediate-sized oligosaccharides (Dp range 7 to 20, average Dp = 13) were eluted with high-salt buffer.

A terminal aldehyde group was generated at the nonreducing end of the intermediate-sized oligosaccharides by an oxidation reaction using 100 mM sodium periodate for 15 to 30 min at ambient temperature in the dark. Excess ethylene glycol was used to quench the reaction and the product was desalted on a Sephadex G-25 column (Pharmacia) in water and lyophilized.

The oligosaccharide-protein conjugate was prepared by stirring a mixture of terminal aldehyde-containing *N*-Pr meningococcal B oligosaccharide with tetanus toxoid (molar ratio of 200:1, respectively) in 0.75 M potassium phosphate buffer, pH 9.0, with sodium cyanoborohydride (40 mg/ml) for 1 day at 40°C, then 2 days at ambient temperature. The resultant *N*-Pr meningococcal B oligosaccharide-tetanus toxoid conjugate was purified by gel permeation chromatography on Sephadex G-100 (Pharmacia) using PBS as the eluting buffer. Sialic acid and protein content of the conjugate vaccine were measured by the Svennerholm resorcinol reaction (21) and Lowry et al. (22) assays, respectively. On a weight basis, the final saccharide to protein ratio of the conjugate was determined to be 0.15. A Western blot employing both anti-tetanus toxoid-specific and anti-*N*-Pr meningococcal B-specific antisera was used to confirm that the protein and saccharide contained in the final product were covalently linked (data not shown).

Preparation of mAbs

Four- to six-week-old female CD1 mice, obtained from Charles River Italia S.P.A. (Calco, Italy), were vaccinated with an *N*-Pr meningococcal B-tetanus toxoid conjugate vaccine prepared as described above. The 0.1-ml dose contained 2.5 μg of sialic acid and 16 μg of tetanus toxoid protein, and was administered i.p. together with CFA. In individual experiments, two or three injections were given, each separated by 1 mo, with the final dose given without adjuvant. Three days later, animals were sacrificed and their spleen cells were fused with myeloma cells P3X63-Ag8.653 at a ratio of 5 spleen cells to 1 myeloma cell. After 2 wk of incubation in HAT-selective medium, hybridoma supernatants were screened for Ab-binding activity to *N*-Pr meningococcal B polysaccharide by an ELISA described below. Specificity of Ab binding was demonstrated by inhibition with high m.w. soluble *N*-Pr meningococcal B polysaccharide (25 μg/ml). Hybridomas secreting reactive Ab were cloned by limiting dilution, and then expanded and frozen for subsequent use in tissue culture, or for ascites production in BALB/c mice.

A total of 46 cell lines were prepared from three fusions. Of the 46 cell lines, the Abs from 30 were characterized. The isotypes of the mAbs were determined using a solid-phase Ab capture ELISA, performed as described below, and alkaline phosphatase-conjugated polyclonal Ab specific for each of the mouse IgG subclasses, IgM, IgA, and κ and λ light chains (Zymed, South San Francisco, CA). Among the 30 Abs, one was an IgM and the remaining 29 were IgG (3 IgG1, 3 IgG2a, 15 IgG2b, and 8 IgG3). All of the Abs had κ light chains. The isotypes and other selected characteristics of these Abs are summarized in Table I.

Measurement of mAb concentration

mAbs were partially purified from tissue culture fluid or mouse ascites by ammonium sulfate fractionation and exhaustive dialysis. The Abs were quantified by an ELISA capture assay performed as previously described (7). In the present study, the "capture" Ab consisted of affinity-purified rabbit anti-mouse IgG, IgM, and IgA (Zymed) diluted to 1 μg/ml in PBS (pH 7.4) and adsorbed to microtiter plates (Immulon 2; Dynatech Laboratories, Chantilly, VA) by incubation overnight at 4°C. Bound mouse mAb was detected using alkaline phosphatase-conjugated rabbit anti-murine IgG, IgM, and IgA polyclonal Abs (Zymed). The Ig concentration of the partially purified mAb preparations were calculated from standard curves prepared from titrations of commercial murine IgG1, IgG2b, IgG3, and IgM standards (Southern Biotechnology Associates, Birmingham, AL) at concentrations ranging from 4 ng/ml to 500 ng/ml. The concentration of selected mAbs also was measured by a commercial radial immunodiffusion assay performed according to the manufacturer's instruction (The Binding Site Limited, Birmingham, U.K.).

Binding to N*-Pr meningococcal B polysaccharide*

A solid-phase ELISA was used to assess the binding of the mAbs to biotinylated *N*-Pr meningococcal B polysaccharide bound to avidin-coated microtiter wells, using a procedure previously described (23). Specificity of antigenic binding was confirmed by demonstration of inhibition of binding in the presence of an excess of soluble *N*-Pr meningococcal B polysaccharide (25 μg/ml). The ability of *N*-Pr meningococcal B oligomers (Dp range of 3 to 6; average = 3.8) to inhibit binding also was determined by a competitive solid-phase ELISA performed as described above, except that the mAbs were prediluted to concentrations to yield an OD of 0.5 to 1.0 after approximately 30 min of incubation with substrate. The mAbs were added to wells of replica plates, each containing a final inhibitor concentration of 25 μg/ml of high m.w. *N*-Pr meningococcal B polysaccharide, or oligomers of *N*-Pr meningococcal B polysaccharide, or buffer alone. The plates were covered and incubated overnight at 4°C. The following day, the contents of the wells were aspirated, and the plates washed five times with washing buffer. Bound Ab was detected by alkaline phosphatase-conjugated rabbit anti-murine IgG, IgM, and IgA polyclonal Ab. Percent inhibition was calculated by comparing absorbance values at 405 nm after 30

Table I. *Characteristics of anti-*N*-Pr meningococcal B polysaccharide mAbs*

Fine Antigenic Specificity Group[a]	SEAM mAb No.	Ig Isotype	ELISA Reactivity to *N*-Pr MenB PS[b]	ELISA Inhibition of *N*-Pr MenB Binding by *N*-Pr MenB OS[c]	ELISA Reactivity to *N*-Ac MenB PS[d]	Binding to Encapsulated *N. meningitidis* group B[e]	Binding to CHP-134 PSA[f]	Bactericidal Activity[g]
I	10	G1,κ	+++	+++	++	0	0	ND
	11	G2b,κ	+++	++	+++	+	++	R
	18	G2b,κ	+++	+++	+++	+	+	R/H
	20	G2b,κ	+/−	++	++	0	0	0
	21	G2b,κ	+/−	+++	++	0	0	0
	26	G2b,κ	++++	+	+++	+	++	R
	28	G2b,κ	++++	++	++	+	+	R
	29	G2a,κ	++++	++	++	+	++	0
	35	G2b,κ	++++	+	+++	+	++	R/H
II	12	G2a,κ	++++	0	++	+	+	R/H
	13	G3,κ	+++	0	+++	+	++	R/H
	14	G2b,κ	++++	0	+++	+	++	R
	15	G2b,κ	++++	0	+++	+	++	R
	16	G2b,κ	+++	0	+	+	i	R
	30	G3,κ	+++	0	+++	+	++	R/H
III	1	G3,κ	+	+	0	0	0	R
	3	G2b,κ	++++	+++	0	+	0	R
	4	G1,κ	++	++	0	i	i	ND
	5	G3,κ	+/−	+	0	+	0	R/H
	7	G3,κ	+	+	0	i	i	R/H
	8	G3,κ	+++	+++	0	+	0	R/H
	17	M,κ	+	+++	0	0	0	0
	19	G2a,κ	++	++	0	0	i	0
	22	G2b,κ	+	++	0	0	i	0
	23	G2b,κ	++	+	0	0	0	0
	48	G2b,κ	+++	+++	0	+	o	R
IV	2	G3,κ	+/−	0	0	+	0	R/H
	6	G3,κ	+/−	0	0	0	i	0
	9	G1,κ	++	0	0	0	i	ND
	24	G2b,κ	++	0	0	+	0	0

[a] Defined by cross-reactivity with *N*-Ac meningococcal B polysaccharide by ELISA and inhibition of anti-*N*-Pr meningococcal B polysaccharide binding by short *N*-Pr meningococcal B oligomers.

[b] Concentration of mAb required to yield an OD of 0.5 by ELISA with *N*-Pr meningococcal B polysaccharide as the solid-phase Ag: +/−, 5-25 μg/ml; +, 1.0-4.9 μg/ml; ++, 0.1-0.9 μg/ml; +++, 0.01-0.09 μg/ml; ++++, <0.01 μg/ml.

[c] Inhibition of Ab binding to *N*-Pr meningococcal B polysaccharide in an ELISA by soluble *N*-Pr meningococcal B oligosaccharides (Dp < 6: average Dp = 3.8): 0, <20% inhibition; +, 21-48% inhibition; ++, 49-74% inhibition; +++, 75-100% inhibition when tested at OD 0.5–1.

[d] Ab binding to *N*-Ac meningococcal B polysaccharide: 0, OD < 0.15; +, OD = 0.15-0.5; ++, OD = 0.5-1.0; +++, OD > 1.0 when tested at 5–25 μg/ml of Ab by ELISA.

[e] Bacterial binding as determined by indirect fluorescence flow cytometry: 0, no detectable binding to encapsulated strains when tested at 100 μg/ml; +, binding to encapsulated strains 8047 and NMB, but not to nonencapsulated strain M7; i, indeterminate (see text).

[f] Binding to polysialic acid on CHP-134 neuroblastoma cells as determined by indirect fluorescence flow cytometry: 0, no binding activity to polysialic acid (PSA) when tested at 100 μg/ml of Ab; ++ binding activity when tested at 10 μg/ml and inhibitable by neuraminidase treatment; +, binding activity detected at 100 but not 10 μg/ml; i, indeterminate is binding activity not inhibitable by neuraminidase treatment.

[g] Complement-dependent bactericidal activity: R/H, activity with both rabbit and human complement; R, activity with rabbit complement, no activity with human complement; 0, no activity with rabbit complement or human complement (also includes Abs only tested with rabbit complement); ND, not done (for IgG1 Abs).

min of incubation with substrate in wells that contained the inhibitor and the corresponding wells without inhibitor.

Cross-reactivity with N*-Ac meningococcal B polysaccharide*

The mAbs were evaluated for their ability to cross-react with the *N*-Ac meningococcal B polysaccharide as demonstrated by direct binding in a solid-phase ELISA. The method used was similar to that described above for the *N*-Pr meningococcal B polysaccharide ELISA, except that *N*-Ac meningococcal B polysaccharide coupled to adipic acid dihydrazide was used as the solid-phase Ag instead of biotinylated *N*-Pr meningococcal B polysaccharide.

Autoreactivity

The 30 mAbs were evaluated for autoreactivity to host polysialic acid expressed by the human neuroblastoma cell line CHP-134 (24) using flow cytometric detection of indirect immunofluorescence. Cells from nearly confluent cultures were collected in 50-ml centrifuge tubes and centrifuged (1000 × *g*). After the supernatant was decanted, 5 ml of blocking buffer (PBS containing 1% (w/v) BSA and 0.1% (w/v) sodium azide) was added to resuspend the cells. The cells were then counted in a hemacytometer and divided into two equal aliquots. One aliquot was incubated for 2 h at ambient temperature with exoneuraminidase (10 U/10^8 cells; Sigma, St. Louis, MO); the other aliquot was treated identically but without enzyme. The exoneuraminidase treatment cleaves the surface polysialic acid and provides a control in the assay for specificity of Ab binding to polysialic acid. After incubation, the cells from each aliquot were distributed among individual reaction tubes so that each tube contained 10^6 cells. To wash the cells, 2 ml of blocking buffer were added to each reaction tube, the tubes were centrifuged (208 × *g*) for 6 min at 20°C, and the supernatant was removed by aspiration. The washed cells were incubated for 2 h on ice in a total volume of 200 μl with either no Ab, or the indicated concentration (usually 10 or 100 μg/ml) of the test or control Ab.

At the end of the 2 h, blocking buffer (2 ml) was added to each reaction tube, and the tubes were centrifuged as before. Following centrifugation, the supernatant was aspirated and the cells incubated for 1 h at ambient temperature with 150 μl of FITC-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (4 μg/ml; Jackson ImmunoResearch, West Grove, PA). After washing with blocking buffer, 400 μl of 0.25% formaldehyde in PBS buffer was added to the cells, and the cells were analyzed by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA).

Control Abs in the assay included: 1) an IgG mAb of irrelevant specificity (VIG10, as a negative control); 2) an IgM anti-polysialic acid mAb (2-1B (25), as a positive control); and 3) an anti-CD56 mAb specific for the protein backbone of neuronal cell adhesion molecule (NCAM; Immunotech, Marseille, France).

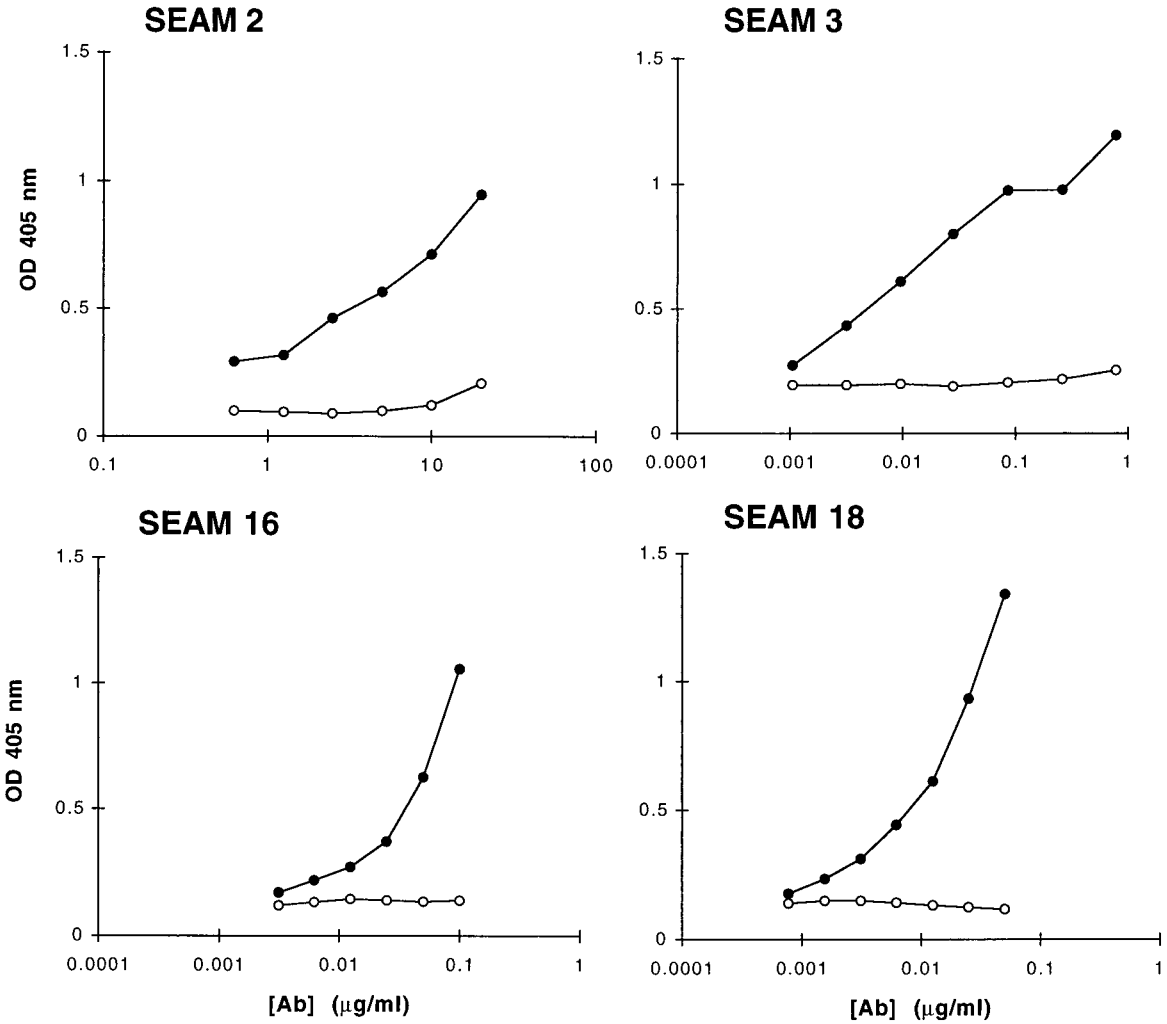

FIGURE 1. Dose-response binding activity of four anti-*N*-Pr meningococcal B polysaccharide mAbs (SEAM 2, 3, 16, and 18, respectively), to solid-phase *N*-Pr meningococcal B polysaccharide as determined by ELISA. Data shown are for the Abs diluted in buffer (●), or in buffer containing 25 μg/ml of soluble *N*-Pr meningococcal B polysaccharide (○). Different ranges for the *x*-axis in the data are used, wherein mAbs SEAM 3, SEAM 16, and SEAM 18 are shown at 0.0001 to 1 μg/ml, and mAb SEAM 2 is shown at 0.1 to 100 μg/ml.

Binding of mAbs to the bacterial surface

The ability of the anti-*N*-Pr meningococcal B polysaccharide mAbs to bind to the surface of pathogenic strains of *N. meningitidis* group B was determined using a flow cytometric detection of indirect immunofluorescence assay. Two fully encapsulated meningococcal B test organisms were used, strain 8047 (the strain used to measure bactericidal activity, see below) and strain NMB. A third, unencapsulated strain, M7, which is a transposon-containing mutant of NMB (26), was used as a negative control for specificity of Ab binding to the encapsulated bacteria. Bacterial cells grown to mid-log phase in Mueller-Hinton broth and 0.25% glucose were harvested and resuspended in blocking buffer at a density of $\sim 10^8$ cells per ml. The mAbs (concentration of 10 or 100 μg/ml) were then added and the mixture was incubated for 2 h on ice. Following two washes with blocking buffer, the cells were incubated with FITC-conjugated $F(ab')_2$ fragment goat anti-mouse IgG (H+L) (Jackson ImmunoResearch), fixed with 0.25% formaldehyde in PBS buffer, and analyzed by flow cytometry.

Positive control Abs included meningococcal-specific serotyping and subtyping mAbs (MN2C3B and MN16C13F4; Rijksinstituut Voor Volksgezondheid en Mileu, Bilthoven, The Netherlands). The negative control consisted of a mouse IgG mAb (VIG10) of irrelevant specificity.

Complement-dependent bactericidal Ab activity

The bactericidal assay was adapted from the method previously described (7), with the following modifications. The test organism, strain 8047, was grown for approximately 2 h in Mueller-Hinton broth containing 0.25% glucose, which rendered the organism more resistant to complement-mediated bacteriolysis by endogenous "natural" Abs, compared with organisms grown in Mueller-Hinton without supplemental glucose (D. M. Granoff, unpublished observations). In the serum bactericidal assay, several complement sources were tested (e.g., human serum, infant rabbit serum; see *Results*). The complement source was used at 20% (v/v) in the final reaction mixture, along with serial twofold dilutions of the mAbs in Gey's buffer containing 1% BSA (instead of barbital buffer as previously described by Mandrell, Azmi, and Granoff (7)). Serum bactericidal titers were defined as the Ab concentration resulting in a 50% decrease in CFU per ml after 60 min of incubation of bacteria in the reaction mixture, compared with control bacteria at the initiation of the incubation period.

Results

Concentration-dependent binding to N*-Pr meningococcal B polysaccharide*

A solid-phase ELISA was used to assess the concentration-dependent binding of the mAbs to *N*-Pr meningococcal B polysaccharide in the presence of dilution buffer alone, or dilution buffer containing 25 μg/ml of soluble *N*-Pr meningococcal B polysaccharide inhibitor. Figure 1 shows data from testing four Abs (SEAM 2, 3, 16, and 18). The concentrations of each of the 30 mAbs sufficient to yield an OD of 0.5 after 30 min of incubation with substrate ranged from less than 0.01 μg/ml to 25 μg/ml (Table I).

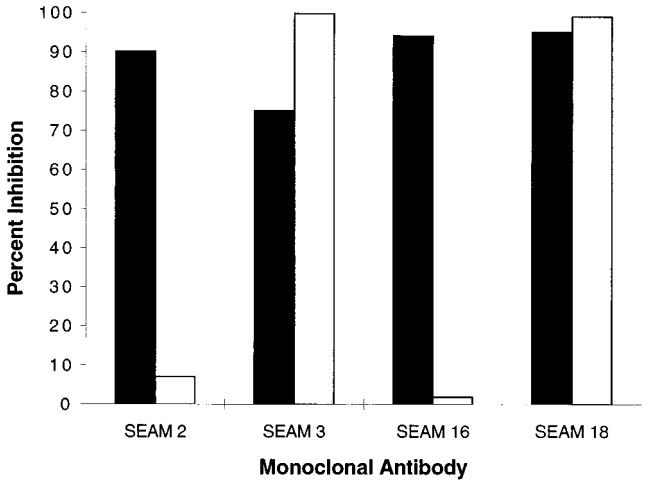

FIGURE 2. Inhibition of binding of four anti-*N*-Pr meningococcal B polysaccharide mAbs (SEAM 2, SEAM 3, SEAM 16, and SEAM 18) to solid-phase *N*-Pr meningococcal B polysaccharide by either 25 μg/ml of soluble high m.w. (HMW) *N*-Pr meningococcal B polysaccharide inhibitor (solid bars), or 25 μg/ml of low m.w. (LMW) *N*-Pr meningococcal B oligosaccharide inhibitor (open bars), as determined by ELISA. The LMW oligosaccharide had a Dp of < 6 (average $= 3.8$).

Fine antigenic specificity

Figure 2 depicts the inhibition of binding of four anti-*N*-Pr meningococcal B polysaccharide mAbs to solid-phase *N*-Pr meningococcal B polysaccharide by either 25 μg/ml of soluble high m.w. *N*-Pr meningococcal B polysaccharide, or 25 μg/ml of soluble *N*-Pr meningococcal B oligosaccharides (Dp < 6; mean $=$ 3.8). There was 75 to 95% inhibition of Ab binding in the presence of the high m.w. polysaccharide of all the mAbs tested. However, as shown in Figure 2, differences in fine antigenic specificity of the different Abs are evident from the results of inhibition studies performed with lower m.w. oligomers. For example, binding of mAbs SEAM 3 and 18 to solid-phase *N*-Pr meningococcal B polysaccharide is completely inhibited by the soluble oligosaccharide, while there is no significant inhibition (less than 20%) of the binding activity of mAb SEAM 2 or 16.

Further heterogeneity in antigenic specificity of the panel of mAbs was evident from studies of their ability to cross-react with *N*-Ac meningococcal B polysaccharide in a direct binding solid-phase ELISA (Fig. 3). Some of the Abs showed strong cross-reactivity, such as mAb SEAM 18, which gave a positive signal when tested at 0.5 μg/ml. Other Abs, such as mAb SEAM 12, were positive only when tested at 10-fold or higher concentrations. In contrast, other Abs, such as mAbs SEAM 2 and 3, were negative when tested at up to 50-fold higher Ab concentrations.

Based on oligosaccharide inhibition of anti-*N*-Pr meningococcal B polysaccharide binding (positive or negative), and cross-reactivity with *N*-Ac meningococcal B polysaccharide (positive or negative), the mAbs could be subdivided into four "fine antigenic specificity subgroups" (designated groups I, II, III, or IV; Table I).

Autoreactivity

The ability of the anti-*N*-Pr meningococcal B polysaccharide mAbs to cross-react with host polysialic acid was assessed by indirect fluorescence flow cytometry. In this assay, the human neuroblastoma cell line CHP-134, which expresses on its surface long-chain polysialic acid associated with NCAM, serves as a cellular marker for human polysialic acid Ags. In a typical experiment (Fig. 4), cells incubated without a primary mAb, or with a control murine mAb having an irrelevant antigenic specificity, show very little fluorescence (approximately 98% of the cells have $<$10 U of fluorescence, *panel A*). In contrast, virtually all cells treated with the anti-*N*-Ac meningococcal B polysaccharide murine mAb, 2-1B, fluoresce strongly (*panel B, left*). This fluorescence is decreased to control levels when the Ab is incubated with cells that had been pretreated with neuraminidase (*panel B, right*). Similarly, cells treated with anti-CD56 fluoresce strongly (*panel C*). With this Ab, the fluorescence is unaffected by pretreatment of the cells with neuraminidase, since the CD56 determinant is located in the protein backbone of NCAM and is unaffected by the removal of polysialic acid with neuraminidase.

The anti-*N*-Pr meningococcal B polysaccharide mAb, SEAM 5, gives no detectable binding when tested at 100 μg/ml (Fig. 4*D*), and is considered as negative in this assay. In contrast, mAb SEAM 35 shows strong polysialic acid-specific binding when tested at 10 or 100 μg/ml (*panels E* and *F*), and is considered positive. A few anti-*N*-Pr meningococcal B polysaccharide mAbs show binding when tested at 100 μg/ml, but appear to be negative when tested at 10 μg/ml (see, for example, mAb SEAM 12 in Fig. 4, *panels G* (10 μg/ml) and *H* (100 μg/ml)). A rare mAb appeared to have weak reactivity that was unaffected by the pretreatment of

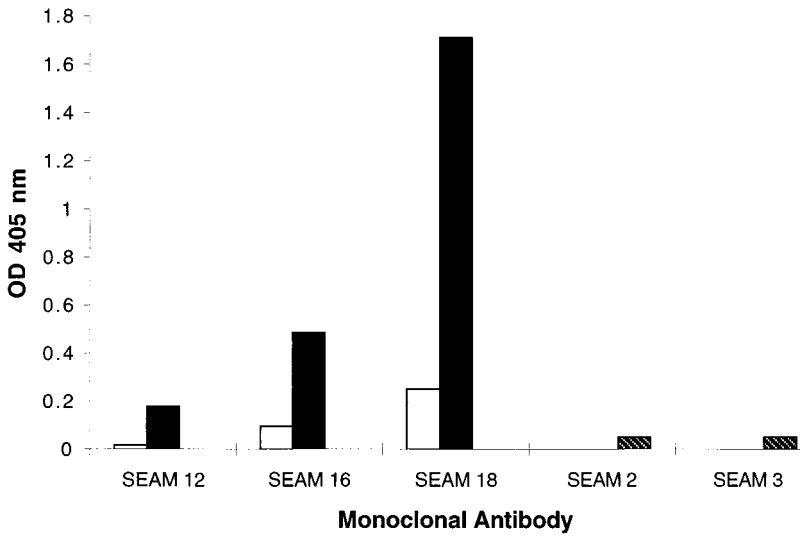

FIGURE 3. Cross-reactivity of five anti-*N*-Pr meningococcal B polysaccharide mAbs (SEAM 12, SEAM 16, SEAM 18, SEAM 2, and SEAM 3) to solid-phase *N*-Ac meningococcal B polysaccharide as determined by ELISA. Three of the Abs, SEAM 12, SEAM 16, and SEAM 18, showed significant binding when tested at 0.5 μg/ml (open bars) and/or 5 μg/ml of Ab (closed bars). Two other Abs, SEAM 2 and SEAM 3, were negative when tested at these concentrations (data not shown) and at fivefold higher concentrations (25 μg/ml of Ab, hatched bars).

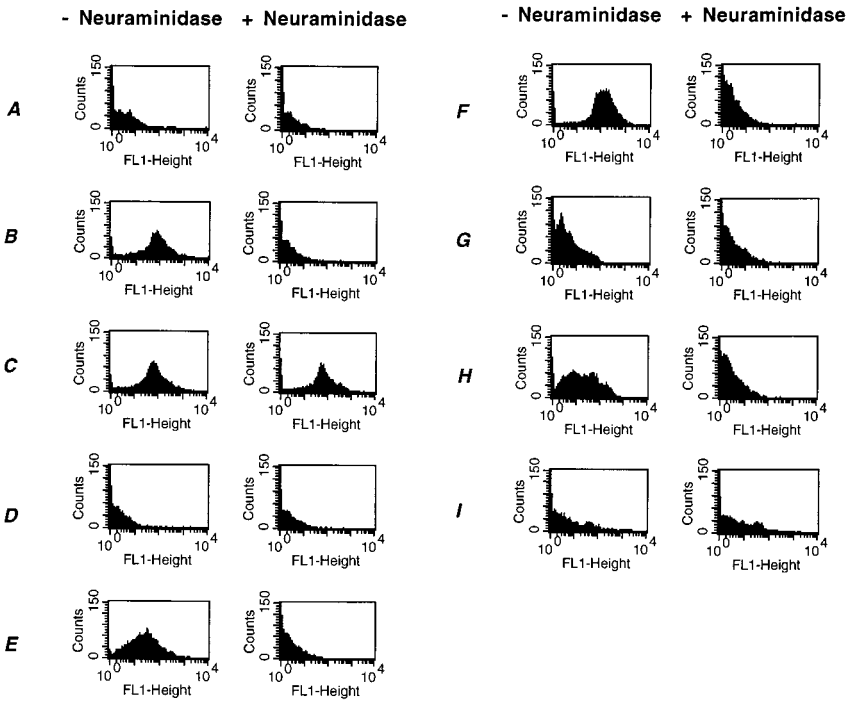

FIGURE 4. Cross-reactivity of representative anti-*N*-Pr meningococcal B polysaccharide mAbs with polysialic acid Ags displayed on the surface of the human neuroblastoma cell line CHP-134 as determined by indirect fluorescence flow cytometry. The following murine mAbs and concentrations tested are shown: *A*, VIG10, negative control (irrelevant specificity), 100 μg/ml; *B*, 2.1B, positive control (anti-*N*-Ac meningococcal B polysaccharide), 5 μg/ml; *C*, neuraminidase control (anti-CD56, see text), 1.5 μg/ml; *D*, SEAM 5, 100 μg/ml; *E*, SEAM 35, 10 μg/ml; *F*, SEAM 35, 100 μg/ml; *G*, SEAM 12, 10 μg/ml; *H*, SEAM 12, 100 μg/ml; and *I*, SEAM 7, 100 μg/ml.

the neuroblastoma cell line cells with neuraminidase (see monoclonal SEAM 7, *panel I*). The autoreactivity of such Abs with polysialic acid was, therefore, considered indeterminate in this assay. The results of autoantibody activity for each of the 30 anti-*N*-Pr meningococcal B polysaccharide mAbs are summarized in Table I.

As shown in Table I, none of the 15 Abs that were negative for binding to *N*-Ac meningococcal B polysaccharide by ELISA (i.e., fine antigenic specificity groups III or IV) was found to bind to polysialic acid in the CHP-134 assay. In contrast, all 11 Abs showing autoantibody activity in the CHP-134 assay also showed cross-reactivity with *N*-Ac meningococcal B polysaccharide in the ELISA (fine antigenic specificity groups I and II). There were four Abs in groups I and II that cross-reacted with *N*-Ac meningococcal B polysaccharide by ELISA that were either negative for autoantibody activity (mAbs SEAM 10, 20, and 21) or were indeterminate (monoclonal SEAM 16). Whether these results reflect insufficient sensitivity of the autoantibody assay, or whether these *N*-Ac meningococcal B cross-reacting Abs truly lack autoantibody activity to host polysialic acid, requires additional study.

Bacterial binding assay

The ability of the anti-*N*-Pr meningococcal B polysaccharide mAbs to bind to the surface of pathogenic strains of *N. meningitidis* group B also was determined using a flow cytometric detection of indirect immunofluorescence assay. As described in *Materials and Methods*, two fully encapsulated meningococcal B test organisms were used, strain 8047 (the strain used to measure bactericidal activity, see below) and strain NMB. A third unencapsulated strain, M7, which is a transposon-containing capsular-deficient mutant of NMB, was used as a negative control for specificity of Ab binding to the encapsulated bacteria.

Figure 5 *A–G* shows the results from a representative bacterial Ab-binding experiment. Anti-*N*-Pr meningococcal B polysaccharide mAbs SEAM 3 and 18 show strong capsular-specific binding to both encapsulated test strains (*panels D* and *E*, respectively). In contrast, mAbs SEAM 9 and 10 were negative in this assay (*panels F* and *G*, respectively). As summarized in Table I, 19 of the 30 anti-*N*-Pr meningococcal B polysaccharide mAbs tested showed evidence of bacterial binding when tested at 100 μg/ml. For each of these, no Ab binding was detected with the unencapsulated mutant. Two additional Abs showed evidence of weak binding to both encapsulated and nonencapsulated mutant strains (see, for example, mAb SEAM 7, as depicted in Fig. 5*H*). Therefore, although these Abs are polysaccharide specific, based on their inhibition in an ELISA (Table I and Fig. 1), their specificity of binding with respect to the bacteria was considered indeterminate. The remaining 9 Abs were negative in the bacterial binding assay.

Complement-mediated bactericidal activity

The percent survival of *N. meningitidis* strain 8047 when incubated with different concentrations of Ab and 20% complement is shown for four mAbs (Fig. 6). Each Ab was tested with three different complement sources: infant rabbit serum pool I, infant rabbit serum pool II, and human agammaglobulinemia serum. For anti-*N*-Pr meningococcal B polysaccharide Abs SEAM 5 (IgG3) and 12 (IgG2a), a similar dose response was observed with each of the three complement sources. In contrast, mAb SEAM 18 (IgG2b) required higher Ab concentrations to elicit bacterial killing in the presence of human complement than were required with either source of rabbit complement. mAb SEAM 3 (IgG2b) showed effective killing when tested with one of the two rabbit complement sources, less effective activity with the second rabbit complement source, and no activity with the human complement source (despite excellent bacterial binding as determined by the flow cytometric immunofluorescence assay; Fig. 5). The ability of each of the mAbs to activate bacteriolysis in the presence of rabbit complement pool 1 and/or human complement is reported in Table I. There were examples of mAbs from each of the four fine antigenic specificity groups that could activate complement-mediated bacteriolysis.

One of the bactericidal mAbs, SEAM 1, had unusual properties in that it had no detectable binding to *N*-Ac meningococcal B

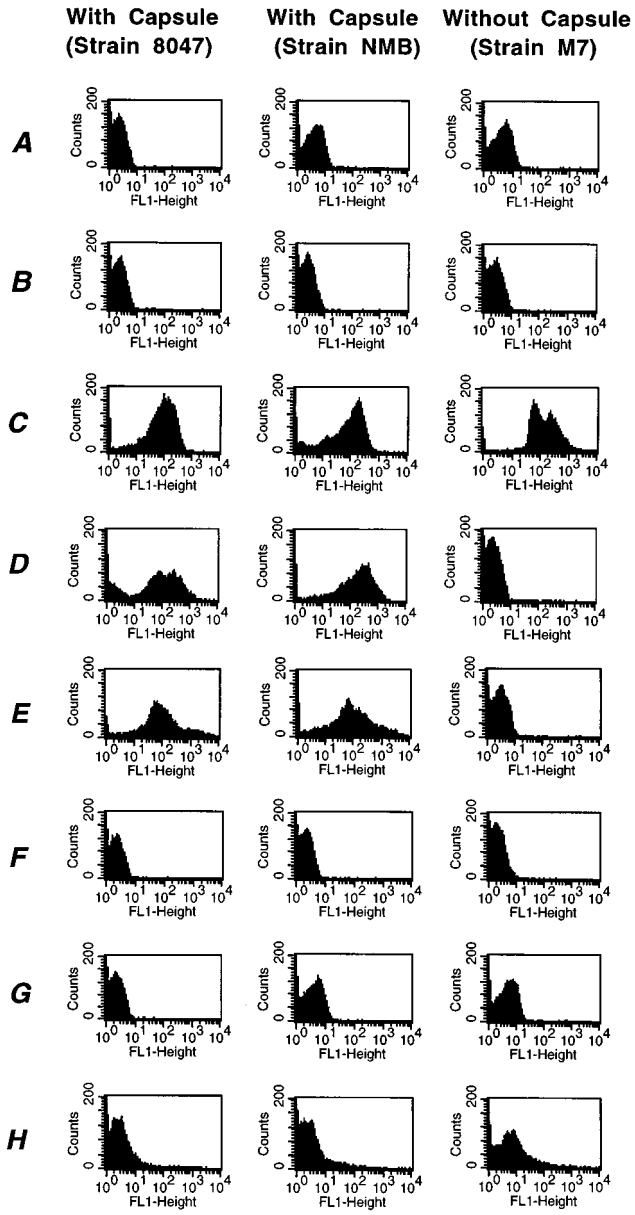

FIGURE 5. Cross-reactivity of representative anti-*N*-Pr meningococcal B polysaccharide mAbs with encapsulated and nonencapsulated whole meningococcal B bacteria as determined by indirect fluorescence flow cytometry. The capsule of strains 8047 and NMB contains *N*-Ac meningococcal B polysaccharide. All mAbs were tested at 100 μg/ml. *A*, Negative control (secondary goat FITC-conjugated anti-murine Ig Ab only); *B*, VIG10, negative control murine mAb (irrelevant specificity); *C*, MN16C13F4, positive control murine mAb for encapsulated and nonencapsulated strains (*N. meningitidis* subtype reagent, P1.2); *D*, SEAM 3 (positive); *E*, SEAM 18 (positive); *F*, SEAM 9 (negative); *G*, SEAM 10 (negative); *H*, SEAM 7 (indeterminate, see text).

polysaccharide by ELISA, and its binding to encapsulated meningococcal B organisms was considered negative in the flow cytometric immunofluorescence assay. Yet this Ab was able to elicit complement-mediated bacteriolysis, although relatively high Ab concentrations were required (46 μg/ml for 50% bacteriolysis). The most likely explanation for the discrepancy in the two assays is that the bactericidal assay is more sensitive for detection of Ab interacting with complement on the bacterial cell surface than is the flow cytometric assay for detecting bound Ab. Note also that, in the bacterial binding assay with SEAM 1, fluorescence was detected above background but the magnitude was considered insufficient to be considered positive in the assay. Two other mAbs, SEAM 2 and 5, required relatively high Ab concentrations to bind to *N*-Pr meningococcal B polysaccharide by ELISA (Table I), but were able to activate bacteriolysis despite their apparent low avidity. However, both of these Abs required relatively high concentrations to elicit bacteriolysis (Ab concentrations for 50% lysis in the presence of rabbit complement of 18 and 35 μg/ml vs 1 to 3 μg/ml for more active Abs such as SEAM 28 or 3).

Discussion

A panel of 30 murine mAbs elicited by immunization with the *N*-Pr derivative of meningococcal B polysaccharide conjugated to tetanus toxoid was produced and characterized. All of the Abs were specific for *N*-Pr meningococcal B polysaccharide based on inhibition of binding to solid-phase Ag by soluble *N*-Pr meningococcal B polysaccharide (i.e., the Abs did not recognize "neoepitopes" created by the conjugation process used to prepare the vaccine). Among the collection there was 1 IgM Ab and 29 IgG Abs. The predominance of IgG Abs was expected, since in contrast to the native meningococcal B polysaccharide, which elicits IgM Abs, conjugate vaccines prepared from the *N*-Pr derivative of the meningococcal B polysaccharide elicit a predominantly IgG Ab response (11, 13, 27). Among the 29 IgG mAbs described in Table I, there was extensive heterogeneity in both the isotype distribution as well as fine antigenic specificities. Differences in fine antigenic specificity were defined by recognition of long or short epitopes, the latter defined by the ability of small *N*-Pr meningococcal B oligosaccharides (Dp $<$ 6) to inhibit Ab binding to *N*-Pr meningococcal B polysaccharide. The mAbs also could be subdivided by their ability to recognize polysialic acid epitopes, as indicated by cross-reactivity with *N*-Ac meningococcal B polysaccharide in an ELISA (Table I). In addition, the Abs differed with respect to their relative avidities for binding to *N*-Pr meningococcal B polysaccharide. For example, approximately 30% required high concentrations to yield positive binding in an ELISA (see for example SEAM 2, Fig. 1), whereas other Abs gave strong positive signals when tested at concentrations as low as 0.01 μg/ml (SEAM 18, Fig. 1). The Abs also differed with respect to their ability to activate complement-mediated bactericidal activity against strains of *N. meningitidis* group B, and autoantibody activity to polysialic acid as assessed by the CHP-134 neuroblastoma cell-binding assay.

The diverse characteristics of the Abs in this panel (Table I) are strikingly different from those described recently by Pon et al. (27). They reported that an *N*-Pr meningococcal B polysaccharide conjugate vaccine evoked a narrow immune response dominated by Abs recognizing a long polysaccharide epitope that did not cross-react with *N*-Ac meningococcal B polysaccharide. Further, they found that only Abs recognizing a long epitope were bactericidal in the presence of complement. In contrast, most (20 of 30) of the mAbs in our panel recognized a short epitope. Further, of the 18 mAbs that were able to mediate bacteriolysis in the presence of complement, 11 were specific for a short epitope. The basis for the different results in our study and the previous study by Pon et al. (27) is not clear. They could relate to the smaller average chain length of saccharide in the conjugate vaccine used to elicit the Abs in our study (average Dp of 13 vs an estimated 35 to 40 in the conjugate vaccine used in the earlier study). The different antigenic specificities also could be a result of the use of different adjuvants in the two studies (CFA vs RIBI adjuvant), or different mouse strains (CD1 vs BALB/c).

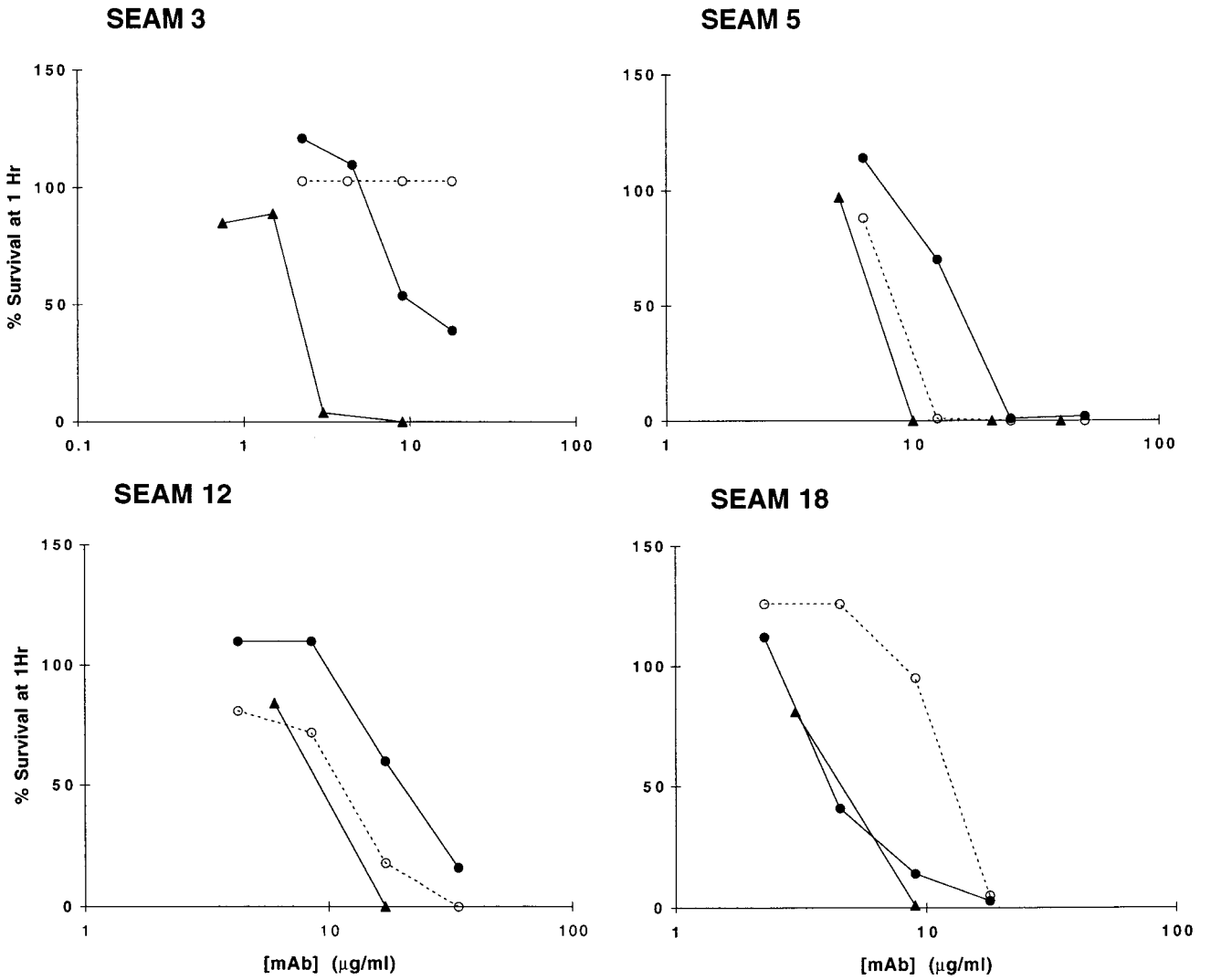

FIGURE 6. Complement-mediated bactericidal activity of four anti-*N*-Pr meningococcal B polysaccharide mAbs (SEAM 3, SEAM 5, SEAM 12, and SEAM 18, respectively) when tested against the meningococcal B test strain 8047. Results are shown from experiments with three different complement sources: infant rabbit complement I (▲); infant rabbit complement II (●); and human complement (○).

Based on their analysis of anti-*N*-Pr meningococcal B mAbs, and on earlier studies of polyclonal sera by the same group (28), Pon et al. suggested that *N*-Pr meningococcal B polysaccharide mimics a unique meningococcal B polysaccharide epitope that is characterized by being "extended" (or long) and possibly involving associated nonpolysaccharide molecules (27, 28). However, in the panel of anti-*N*-Pr meningococcal B mAbs described here, there was no correlation between any particular fine antigenic specificity (short or long epitopes, *N*-Ac meningococcal B polysaccharide cross-reactive or noncross-reactive) and the ability of the Ab to activate complement-mediated bactericidal activity. Further, in other studies not presented, some of the bactericidal mAbs recognizing short segments, such as mAbs SEAM 3 and 18, also have been shown to have opsonic activity for *N. meningitidis* group B organisms in vitro (A. F. M. Verheul, H. Snippe, and B. Bernaissa-Trouw, Utrecht University, The Netherlands, personal communication). The SEAM 18 mAb also has been shown to confer passive protection against meningococcal B bacteremia and meningitis in infant rats (H. Käyhty et al., National Public Health Institute, Helsinki, Finland, personal communication). Taken together, the present data provide proof that there is more than one epitope on *N*-Pr meningococcal B polysaccharide that can mimic protective epitopes on the bacterial surface of encapsulated *N. meningitidis* group B, and that these *N*-Pr meningococcal B polysaccharide epitopes can exist in short or long saccharide segments.

A second important finding of the present study was that 11 of the 18 mAbs that mediated bactericidal activity had autoantibody activity defined by their cross-reactivity with human polysialic acid. This group of autoantibodies included representatives from fine antigenic specificity groups I and II that recognized short or long epitopes, respectively. Virtually all Abs positive for autoantibody activity cross-reacted with *N*-Ac meningococcal B polysaccharide in an ELISA. This correlation was expected, since the structure of the meningococcal B polysaccharide and host polysialic acid expressed by this cell line are reported to be the same (i.e., $\alpha(2\rightarrow8)$ *N*-Ac neuraminic acid (9)). Although the Ab repertoire in response to *N*-Pr meningococcal B polysaccharide conjugate vaccines may be variable in different animal strains given different vaccine compositions, the present data underscore the potential of protective epitopes on this derivatized polysaccharide to elicit autoantibodies. Despite the apparent safety of a prototype *N*-Pr meningococcal B conjugate when tested in subhuman primates (14), the present autoantibody results raise concerns about the safety of such vaccines for human use.

Finally, for the purpose of developing anti-meningococcal B vaccines based on molecular mimetics, Abs that are bactericidal in the presence of complement, but that do not cross-react with human polysialic acid Ags, are of particular interest for Ag selection. Of the 30 anti-*N*-Pr meningococcal B polysaccharide mAbs studied, eight Abs (SEAM 1, 2, 3, 5, 7, 8, 16, and 48) exhibited these desired characteristics. A second group of three mAbs (SEAM 12, 18, and 28) were weakly cross-reactive with polysialic acid and may also be useful for identifying molecular mimetics of unique meningococcal B polysaccharide epitopes. This collection of 11 mAbs includes representatives from each of the 4 fine antigenic specificity groups and of 3 isotypes (IgG2a, IgG2b, and IgG3).

Vaccines containing molecular mimetics of *N*-Pr meningococcal B polysaccharide may be capable of eliciting protective Abs against meningococcal B disease with minimal risk of autoantibody activity. The underlying principle that a mimetic can elicit a protective anti-polysaccharide Ab response was demonstrated by Westerink et al. (18) and Agadjanyan et al. (29). In the studies of Westerink et al., they prepared a vaccine from a small peptide having an amino acid sequence corresponding to a loop segment of a murine anti-idiotypic Ab. The anti-idiotypic Ab had been prepared against an anti-meningococcal C polysaccharide mAb and had been shown to be a mimetic of group C meningococcal polysaccharide (30). The isolated peptide vaccine also was able to elicit an Ab response against meningococcal C polysaccharide, and protected mice against a lethal challenge by serogroup C meningococci. The bactericidal, nonautoreactive *N*-Pr meningococcal B polysaccharide mAbs described here might be useful for preparing anti-idiotypic Abs and applying this approach to the development of a safe meningococcal B vaccine. Alternatively, the mAbs could be useful in screening combinatorial libraries such as phage display peptide libraries (31) or combinatorial molecule pools to identify mimetics of unique meningococcal B epitopes. These possibilities are currently being investigated.

Acknowledgments

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