Antibody Responses to the Capsular Polysaccharide of *Neisseria meningitidis* Serogroup B in Patients with Meningococcal Disease

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We measured antibody responses to meningococcal serogroup B (MenB) polysaccharide (PS) by enzyme-linked immunosorbent assay (ELISA) in sera from 94 patients from The Netherlands with disease caused by *Neisseria meningitidis* group B. The patients ranged in age from 3 to 73 years (mean age, 18.8 years). In initial studies we showed that the binding of a panel of MenB PS-reactive human immunoglobulin M (IgM) paraproteins to biotinylated MenB PS bound to avidin-coated microtiter wells was inhibited >90% by the addition of soluble MenB PS or encapsulated group B meningococci. In contrast, inhibition of IgM anti-MenB PS antibody-binding activity in many of the patient sera was less than 50% (range, 20 to 94%). These data suggested a high frequency of nonspecific binding in the patient sera. Therefore, all serum samples were assayed in replicate in the presence or absence of soluble MenB PS, and only the inhibitable fraction of the binding signal was used to calculate the anti-MenB PS antibody concentrations. In 17 control patients with meningococcal disease caused by serogroup A or C strains, there was no significant difference in the respective IgM or IgG anti-MenB PS antibody concentrations in paired acute- and convalescent-phase sera. In contrast, in patients with MenB disease, the geometric mean IgM anti-MenB PS antibody concentration increased from 3.9 units/ml in acute-phase serum to 10.5 units/ml in convalescent-phase serum ($P < 0.001$). The corresponding geometric mean IgG anti-MenB PS antibody titers were 1:27 and 1:36 ($P < 0.05$). There was only a weak relationship between age and the magnitude of the logarithm of the antibody concentrations in convalescent-phase sera (for IgM, $r^2 = 0.06$ and $P < 0.05$; for IgG, $r^2 = 0.08$ and $P < 0.01$). Our data indicate that precautions are needed to avoid nonspecificity in measuring serum antibody responses to MenB PS by ELISA. Furthermore, although this PS is thought to be a poor immunogen, patients as young as 3 years of age recovering from MenB disease demonstrate both IgM and IgG antibody responses in serum.

Serogroup B strains of *Neisseria meningitidis* are an important cause of meningitis and sepsis in many areas of the world (5, 10, 26). The incidence of disease is highest in infants, but disease can occur in all age groups (4). Polysaccharide (PS) vaccines have been developed for protection against diseases caused by meningococcal serogroups A, C, Y, and W135 (21, 24). These vaccines are effective in adults, but they are less effective in infants and toddlers (8, 19, 20, 31, 34, 39, 42).

No licensed vaccines are available in the United States or Europe to protect against *N. meningitidis* serogroup B disease. Experimental serogroup B vaccines that induce serum antibodies to either outer membrane proteins or the meningococcal group B (MenB) PS have been tested in children and adults (6, 11, 15, 16, 43, 52, 54). MenB vaccines based on outer membrane vesicles have limited effectiveness in infants and have the potential disadvantage of serotype specificity (11, 15). MenB PS-based vaccines also have a number of drawbacks. Serum anti-MenB antibodies elicited by vaccination or invasive meningococcal disease are reported to be of low magnitude, pre-

dominantly immunoglobulin M (IgM) (33, 36, 48, 54), and of low avidity (36). Also, there are safety concerns: anti-MenB PS antibodies are autoantibodies in that they cross-react with polysialic acid residues [i.e., $\alpha(2 \rightarrow 8)$ -linked *N*-acetylneuraminic acid residues] expressed in fetal tissue and some adult tissues (13, 32, 44). Indeed, one reason for the poor immunogenicity of MenB PS may be immunologic tolerance induced by exposure to host polysialic acids that are structurally similar to MenB PS (13).

Limited information on the variable region gene repertoire of human anti-MenB PS antibody responses is available. In a previous study (2), our laboratory investigated idiotypic expression and the heavy and light chain Ig variable (V) region gene (VH and VL genes, respectively) usage of a human monoclonal antibody (MAb), MAb 5E1, reactive with MenB PS (2). The VL and VH genes used were highly homologous to the V genes encoding human antibodies to the *Haemophilus influenzae* type b PS and antibodies reactive with self-antigens such as erythrocyte i, DNA, and thyroid peroxidase. These data indicated that this anti-MenB PS antibody is encoded by V regions that recur in the repertoires of human antibodies to both MenB PS and structurally dissimilar PS and autoantigens.

The studies described above were limited to a single human MenB PS-reactive MAb. With the long-term goal of extending these studies to investigate the V-region repertoire of serum anti-MenB-PS antibodies, we developed an enzyme-linked im-

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TABLE 1. Description of serum samples from patients recovering from invasive meningococcal disease

Characteristic	Patients with group B disease (n = 94)	Patients with group A or C disease (n = 32)
Patient age (yr)		
Range	3–73	3–73
Mean	18.8	20.5
No. of samples		
Paired acute and convalescent phase	37	17
Convalescent phase only	57	15
Interval (days) between onset of disease and acquisition of serum sample ^a		
Acute-phase samples	2.0 ± 1.2 (0–4)	4.5 ± 2.4 (1–9)
Convalescent-phase samples (paired)	34.1 ± 15.1 (12–69)	31.7 ± 11.3 (17–54)
Convalescent-phase samples (unpaired)	37.7 ± 18.7 (16–111)	27.2 ± 8.5 (10–38)

^a Values are means ± standard deviations (ranges).

munosorbent assay (ELISA) to measure human anti-MenB PS antibody concentrations. We report here the use of this assay to quantify the serum IgM and IgG anti-MenB antibody responses of patients recovering from invasive meningococcal disease. In previous studies, antibodies to $\alpha(2\rightarrow8)$ -linked *N*-acetylneuraminic acid, the capsular PS of *N. meningitidis* group B and *Escherichia coli* K1, were measured in serum by various serological assays (1, 7, 12, 16, 17, 23, 30, 33, 36, 54, 55). In general, those studies were limited to small groups of healthy individuals or patients with invasive MenB disease. Also, with one exception (12), the assays used did not use a reference anti-MenB PS antibody to permit comparison of the results of different laboratories, and as described further below, the studies that used ELISA may have overestimated serum anti-MenB PS antibody concentrations because of failure to take into consideration nonspecific antibody binding.

MATERIALS AND METHODS

Serum samples. Serum samples from 94 patients from The Netherlands with culture-proven invasive MenB disease were analyzed (Table 1). These samples were obtained between 1989 and 1990 as part of a nationwide study of meningococcal disease in Holland (47). Since a variety of assays of the collection was planned, samples were selected for the present study on the basis of the availability of >1-ml volumes. Paired acute- and convalescent-phase serum samples were available from 37 patients; for the remaining 57 patients, only convalescent-phase serum samples were available. The 94 patients ranged in age from 3 to 73 years (mean age, 18.8 years). Sera from patients younger than 3 years of age were not available because of insufficient volumes.

Control sera were obtained from 32 patients from The Netherlands with culture-proven MenA or MenC disease (Table 1). The control patients with MenA or MenC disease ranged in age from 3 to 73 years (mean age, 20.5 years). Paired acute- and convalescent-phase samples were available from 17 of these patients, and convalescent-phase samples only were available from the remaining 15 patients. Control serum samples were obtained from 43 healthy adults (20 serum samples from Dutch military personnel [ages, 17 to 22 years] obtained in 1990 on their third day after enlistment and 23 serum samples from young adults working in the laboratory at Oakland, Calif.).

Human MAbs. Human IgM(κ) anti-MenB PS MAbs 5E1 and 9B10 derived from Epstein-Barr virus-transformed B-cell lines were provided by Howard Raff, Bristol Myer Squibb, Seattle, Wash. (40, 41). NOV, a MenB PS-reactive IgM(λ) macroglobulin, was provided by Elvin Kabat, Columbia University, New York, N.Y. (29). MenB PS-reactive paraproteins HUFF and LOG came from the collection of Hans Spiegelberg, University of California, San Diego (3).

Anti-MenB PS ELISA. MenB PS (lot FL030283) was provided by Patrick McVerry, Connaught Laboratories, Inc., Swiftwater, Pa. Biotinylated MenB PS

was prepared as described previously (2, 50) by derivatizing with adipic hydrazide by the carbodiimide method (25); this was followed by biotinylation with the *N*-hydroxysuccinimide ester of biotin (45). The final antigen preparation contained 540 nmol of biotin per mg of MenB PS.

Wells of microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, Va.) were incubated overnight at 4°C; each well contained 100 μ l of hen's egg avidin (4 μ g/ml; ExtrAvidin; Sigma) in 10 mM sodium phosphate-buffered saline (PBS; pH 7.40). After washing three times with PBS, 100 μ l of biotinylated MenB PS, diluted 1:5,000 in PBS, was added to each well and the plates were incubated for 2 h at 37°C. The plates were washed three times with PBS, and the wells were filled with blocking buffer (20 mM Tris-buffered saline [TBS] containing 1% bovine serum albumin [BSA] [pH 7.4]) and were incubated for at least 30 min at room temperature to block nonspecific binding sites. The plates were washed three times with washing buffer (TBS, 0.1% Tween 20 [pH 7.40]). Dilutions of test sera of 50 μ l were added to wells of replicate plates containing 50 μ l of diluting buffer (TBS containing 1% BSA and 0.1% Tween 20 [pH 7.40]) or 50 μ l of diluting buffer containing 50 μ g of soluble MenB PS per ml (final inhibitor concentration, 25 μ g/ml). This concentration of inhibitor was selected because in previous studies it resulted in $\geq 95\%$ inhibition of binding of a series of human MenB PS-reactive paraproteins and MAbs (2, 3). The plates were then incubated overnight at 4°C. On the following day, the wells were washed five times in cold washing buffer and were incubated for 3 h at 4°C with 100 μ l of alkaline phosphatase-conjugated murine anti-human antibody per well. For the detection of IgM, a 1:6,000 dilution of alkaline phosphatase-conjugated murine MAb HP6083 was used (the antibody was provided by George Carlone, Molecular Biology Department, Centers for Disease Control and Prevention, Atlanta, Ga., and the conjugate was prepared by American Qallex, La Mirada, Calif.). The plates were then washed with cold washing buffer, and 100 μ l of freshly prepared substrate (substrate 104; Sigma) diluted to 1 mg/ml in substrate buffer (1.0 M diethanolamine, 0.5 mM MgCl₂ [pH 9.8]) was added to each well. Absorbance values were taken when the wells containing 0.025 μ g of IgM 5E1 MAb per ml reached an optical density (OD) of 1.0 to 1.2 (approximately 1 h) (2).

For each test serum sample, a titration curve was obtained by plotting the absorbance values as a function of the logarithm of the reciprocal dilution of the test serum sample. Only absorbance values in the linear portion of the standard curve (0.15 to 1.2) were used to calculate concentration. Absorbance values for wells containing dilutions of serum incubated with soluble MenB PS were subtracted as background from the corresponding values for the wells in which sera were diluted with buffer alone. Only sera with absorbance values that were both inhibited at least 20% by soluble MenB PS and for which the difference between the respective nonabsorbed and absorbed ODs was at least 0.15 were considered positive. The standard curve for the IgM ELISA consisted of dilutions of the human MenB PS-specific IgM MAb, MAb 5E1. This antibody has been extensively characterized with respect to its antigen specificity, functional protective activity, idiotype expression, and VL and VH region genes (2, 40). Antibody concentrations in the test sera were expressed in units of MenB PS antigen-binding equivalence of IgM 5E1 MAb per milliliter. A unit of IgM serum antibody represents ~ 1 μ g of MenB PS-binding activity. However, if differences exist between the avidity of the IgM 5E1 MAb and the anti-MenB PS antibody in a test serum sample, then a unit of antigen-binding activity in a test serum sample will represent a quantity higher or lower than 1 μ g of antibody per ml on a mass basis. Test sera were assayed beginning at a 1:50 dilution. Therefore, the minimum detectable serum IgM antibody concentration was 0.3 unit/ml. Because of day-to-day variability of background absorbance values of samples with low antibody concentrations, 1.0 unit/ml was the minimum IgM antibody concentration detectable in all assays.

For the detection of IgG, a 1:15,000 dilution of alkaline phosphatase-conjugated MAb HP6043 was used (the antibody was provided by George Carlone, Molecular Biology Department, Centers for Disease Control and Prevention, and the conjugate was prepared by American Qallex). Absorbance values were taken when the wells containing a 1:80 dilution of IgG 5E1 MAb reached an OD of 1.0 to 1.2 (see below). Because of the observed tendency of the IgG 5E1 MAb to aggregate, IgG ELISA results were not expressed in units of IgG antibody binding per milliliter, since such results would not be reflective of true monomeric IgG binding. Instead, titers were determined graphically as the intercept of the endpoint of an OD of 0.2, a value that was selected because it was in the linear range of the assay and sufficiently above the background value to ensure the reproducibility of our results.

Two convalescent-phase serum samples from patients with culture-proven MenB disease were used as positive controls: serum sample 23754 for the IgM antibody assay and serum sample 175C for the IgG assay. In nine assays, the IgM anti-MenB PS geometric mean antibody concentration of serum sample 23754 was 12.9 units/ml (95% confidence interval, 11.0 to 13.4 units/ml). The IgG anti-MenB PS antibody titer of serum sample 175C in five assays was 1:161 (95% confidence interval, 137 to 185). A third control was a convalescent-phase serum sample from a patient with MenC disease (serum sample 23755), which contained ~ 1.0 unit of IgM anti-MenB PS antibody per ml (95% confidence interval, 0.84 to 1.25 units/ml) and had no detectable IgG anti-MenB PS antibody (titer, <1:50).

Inhibition of anti-MenB PS binding. Soluble MenB PS was compared with group B encapsulated *N. meningitidis* bacteria for the ability to inhibit the binding

of MenB PS-reactive antibodies to solid-phase MenB PS in an ELISA. Dilutions of the IgM 5E1 MAb or test sera were incubated in the presence of 25 μ g of soluble MenB PS per ml, $\sim 4 \times 10^7$ CFU of *N. meningitidis* group B bacteria (strain NMB [49]) per ml, or buffer alone. Additional specificity controls included test samples incubated with 25 μ g of *N. meningitidis* group C capsular PS per ml or a nonencapsulated mutant (strain M7) of the encapsulated strain *N. meningitidis* NMB described previously (49). In separate experiments, samples of the IgM 5E1 MAb or test sera were preincubated separately with alum-bound MenB PS or MenC PS as inhibitors (see below). The absorbance values were used to calculate the respective percent inhibitions for each of the inhibitors.

Bacterial growth. On the day before the inhibition ELISA was performed, a portion of frozen stock bacteria was incubated at 37°C overnight on GC agar containing supplements as described previously (46, 51). Organisms were collected on a Dacron swab and were suspended in sterile Gey's balanced salt solution to obtain an OD of ~ 0.6 ; a 1:10 dilution of this suspension in diluting buffer was then prepared for use as an inhibitor in the ELISA. To determine the concentration of the bacterial suspension used, a colony count was performed by dilution in Gey's balanced salt solution and plating of the organisms as described previously (53).

Preparation of alum-bound PSs. Purified MenB PS or MenC PS was coupled noncovalently to alum by a modification of a method described previously (18). In brief, aluminum hydroxide gel was prepared by neutralizing a 50 mM AlCl_3 solution to pH 7.00 with 1 M NaOH. The precipitate was pelleted by centrifugation at 400 to 600 $\times g$ for 10 min, and the precipitate was resuspended in TBS. Equal volumes of alum gel suspension were mixed with MenB or MenC PS (1 mg/ml), and the mixtures were incubated for 30 min at room temperature. The alum-PS mixture was then washed three times in ELISA blocking buffer (see above). After washing, the alum-PS gel was diluted 1:10 in ELISA diluting buffer. An equal volume of diluted serum was added to the alum-PS gel, and the mixture was incubated for 2 h at 4°C. The tubes were then microcentrifuged for 2 min, and the supernatant was removed and assayed by ELISA for anti-MenB PS antibody.

Statistical analysis. Antibody concentrations were transformed logarithmically. For IgM, antibody concentrations less than the minimum concentration detectable in the assay (1.0 unit/ml) were assigned values of 50% of the minimum (0.5 unit/ml). For IgG, titers of $<1:50$ were assigned values of 1:25 for calculation of geometric mean titers. For groups of patients with paired acute- and convalescent-phase samples, the difference of the respective logarithms of the acute- and convalescent-phase serum antibody levels was compared by a paired *t* test. The respective logarithms of the antibody concentrations or titers of the acute-phase samples from patients with MenA or MenC disease and those from patients with MenB disease were compared with the logarithms of the antibody concentrations or titers of the samples from the army recruits by independent *t* tests and by regression analysis, adjusting for age. The relationship between age and the logarithms of the antibody concentrations of convalescent-phase serum samples from patients with MenB disease was analyzed by regression.

RESULTS

Specificity of the anti-MenB PS ELISA. In preliminary studies, the specificity of the anti-MenB PS ELISA was evaluated by incubating dilutions of the anti-MenB PS human IgM 5E1 MAb with buffer containing 50 μ g of soluble MenB PS, or soluble MenC PS, or buffer alone per ml (final inhibitor concentration, 25 μ g/ml) to determine the extent of inhibition of anti-MenB PS-binding activity. This concentration of inhibitor was selected on the basis of previous data showing that 25 μ g of MenB PS per ml was in excess of that needed to inhibit $\geq 95\%$ of the binding of a panel of human IgM MenB PS-reactive paraproteins or MAbs (2, 3). MenB or MenC PS inhibition was also performed with selected acute- or convalescent-phase sera from patients with MenB disease. Incubation with excess soluble MenB PS, but not MenC PS, inhibited the binding of anti-MenB PS MAb 5E1 to solid-phase MenB PS by $\geq 98\%$ (Fig. 1A). However, as illustrated in Fig. 1B and C, inhibition of the binding activity of IgM antibody in different patient serum samples by soluble MenB PS varied from 94% (convalescent-phase serum sample 227C) to 20% (acute-phase serum sample 383A). Inhibition values were generally greater in convalescent-phase sera with high antibody concentrations than in acute-phase sera with low antibody concentrations. In none of the serum samples was there significant inhibition of binding to solid-phase MenB PS by soluble MenC PS.

Because of lower than anticipated MenB PS inhibition of antibody binding in some serum samples, we also tested inhi-

bition using a suspension of *N. meningitidis* group B whole bacteria (strain NMB) since it was possible that encapsulated bacteria might express conformational PS epitopes that are not expressed by the soluble PS. However, as shown by the examples in Fig. 1A to C (panels in the right column), the percent inhibition by *N. meningitidis* group B encapsulated bacteria was nearly identical to that observed when soluble MenB PS was used as the inhibitor (Fig. 1A to C, panels in the middle column). The addition of a capsule-deficient strain of group B *N. meningitidis*, strain M7, did not inhibit IgM anti-MenB PS-binding activity (Fig. 1A to C, panels in the right column).

The ability of soluble MenB PS to inhibit binding of IgM MenB PS-reactive antibodies in the ELISA was examined further by using a second human anti-MenB PS MAb, MAb 9B10 (41), and a panel of seven human serum samples containing MenB PS-reactive IgM paraproteins (3). We selected dilutions of antibody that, in the absence of the inhibitor, gave ODs of 0.6 to 1.2 after 1 h of incubation with substrate. The absorbance values obtained with the two IgM MAbs, MAbs 5E1 and 9B10, and three representative IgM paraproteins are given in Fig. 2. For all nine antibodies tested, binding activity was inhibited 92 to 100% by the addition of excess soluble MenB PS. This high level of uniform inhibition suggests that when assaying anti-MenB PS antibody in test sera, noninhibitable signal likely represents nonspecific antibody binding. For this reason, all subsequent assays of MenB PS binding activity in test sera were performed in replicate in the presence or absence of soluble MenB PS, and only the inhibitable signals (i.e., the difference in OD between replicate wells containing dilutions of serum incubated in the presence of buffer alone and dilutions of serum incubated in the presence of buffer containing 25 μ g of MenB PS per ml) were used to calculate the concentrations of anti-MenB PS antibody in serum.

The effect of using the inhibitable signal instead of the total signal in the ELISA to calculate serum IgM anti-MenB PS antibody concentrations is illustrated by the results of assays of 13 representative acute- or convalescent-phase serum samples from patients with MenB disease (Table 2). For each serum sample, antibody concentrations were calculated on the basis of the total binding signal in the absence of an inhibitor or from the inhibitable fraction of antibody binding when the sample was assayed in the presence of soluble MenB PS. In addition, a third antibody concentration was derived for eight of the samples after inhibition of anti-MenB PS binding by MenB PS bound to alum. (The negative control for alum absorption was MenC PS bound to alum; see Materials and Methods.) The alum-MenB PS absorption method was evaluated because of a previous report that MenB PS bound to alum is a more efficient inhibitor of anti-MenB PS antibody binding than soluble MenB PS in an ELISA (33). With soluble MenB PS, inhibition of the patient sera varied from 20 to 94%. With MenB PS bound to alum, in contrast to the previous report (33), we obtained inhibition values very similar to those obtained when soluble MenB PS was used as the inhibitor. Use of the inhibitable binding signals to calculate the anti-MenB PS antibody concentrations for the sera produced antibody concentrations that were lower than those calculated by using the respective uninhibited signal (up to fourfold lower, e.g., for serum sample 383A; 4.7 versus 22.2 units/ml). Thus, in the absence of correction for the noninhibitable fraction, these samples would have been assigned artificially high anti-MenB PS antibody concentrations. Note also that although the data presented here are for IgM anti-MenB PS antibody, in other studies binding of serum IgG antibodies to solid-phase MenB PS was even less inhibitable than binding of the IgM antibodies by soluble MenB PS or encapsulated bacteria (acute-phase

FIG. 1. IgM anti-MenB PS antibody-binding titration curves of human IgM 5E1 MAb and selected acute-phase (dashed lines) and convalescent-phase (solid lines) sera from patients with MenB disease. Serial dilutions of MAb 5E1 or sera from patients with MenB disease were added to replicate wells that contained either diluting buffer alone (panels in the left column) or buffer plus soluble PS (panels in the middle column; ●, with MenC PS; ○, with MenB PS) or buffer plus whole bacteria (panels in the right column; ●, with capsule-deficient group B mutant; ○, with group B bacteria). After incubation overnight at 4°C, bound human IgM was detected by alkaline phosphatase-conjugated murine anti-human IgM MAb HP6083.

sera of subject 212 [Fig. 3B] and acute- and convalescent-phase sera of patient 211 [Fig. 3C]). Therefore, MenB PS inhibition was used to control for nonspecificity in our assays of both serum IgM and IgG anti-MenB antibody concentrations.

Serum anti-MenB PS antibody responses of patients with MenB disease. Serum samples from 94 patients from The Netherlands with culture-proven invasive MenB disease were analyzed for their IgM anti-MenB PS antibody concentrations. Among the 37 patients from whom paired samples were obtained, the geometric mean antibody concentration increased from 3.9 units/ml in acute-phase sera to 10.5 units/ml in convalescent-phase sera (Table 3; $P < 0.001$). The geometric mean fold increase was 2.7 (range, 0.4 to 25.8). Among the 57 patients from whom convalescent-phase serum samples only were obtained, the geometric mean IgM anti-MenB PS antibody concentration was 10.4 units/ml (Table 3). The proportions of all of the convalescent-phase samples (paired and unpaired) with IgM anti-MenB PS antibody concentrations of >2 , >10 , and >25 units/ml were 95, 49, and 21%, respectively.

Figure 4A and B shows the respective acute- and convalescent-phase IgM antibody concentrations for the 37 patients

FIG. 2. Inhibition of binding of human IgM MenB PS-reactive MAb or paraproteins to solid-phase MenB PS by soluble MenB PS. Inhibition was performed with 25 μ g of soluble MenB PS per ml. Dilutions of antibody were selected such that, in the absence of the inhibitor, they gave ODs of 0.6 to 1.2 after 1 h of incubation with substrate.

TABLE 2. IgM anti-MenB PS antibody concentrations in selected acute- and convalescent-phase serum samples calculated in three ways

Serum sample ^a	IgM anti-MenB PS antibody concn (units/ml) calculated on the basis of:		
	Uninhibited ELISA ^b	Soluble MenB PS-inhibited binding ^c	Alum-absorbed MenB PS-inhibited binding ^d
215A	14.0	3.9	3.3
215C	25.8	7.9	6.4
227A	6.5	5.2	Not assayed
227C	111.0	94.4	92.6
296C	29.6	11.9	9.2
325C	6.7	3.9	Not assayed
341A	6.8	2.5	1.3
341C	8.1	3.2	2.4
354C	33.2	9.0	6.9
357C	49.5	44.8	46.7
383A	22.2	4.7	Not assayed
383C	7.0	3.6	Not assayed
385C	11.1	5.9	Not assayed

^a Acute-phase serum samples are indicated by the suffix A, and convalescent-phase samples are indicated by the suffix C.

^b Serum dilutions incubated in the presence of buffer alone.

^c Serum dilutions incubated in the presence of buffer alone or buffer containing 25 µg of soluble MenB PS per ml. Only the fraction of antibody binding that was inhibitable was used to calculate the serum antibody concentrations (see Materials and Methods).

^d Serum dilutions were preabsorbed in MenB PS-alum gel or MenC PS-alum gel for 2 h; this was followed by centrifugation and the addition of the supernatant to the wells of a microtiter plate. Only the fraction of antibody that was inhibitable by MenB PS-alum gel was used to calculate the serum antibody concentrations.

TABLE 3. IgM anti-MenB PS antibody responses of patients recovering from invasive meningococcal disease

Patient group	No. of samples	Log ₁₀ units/ml (mean ± SD) ^a	Geometric mean concn (units/ml)	% Sera with titers of >2 units/ml ^b
Serogroup B disease				
Paired samples				
Acute phase	37	0.59 ± 0.62	3.9 ^c	68
Convalescent phase	37	1.02 ± 0.50	10.5 ^d	97
Unpaired convalescent phase	57	1.02 ± 0.47	10.4 ^e	93
Serogroup A or C disease				
Paired samples				
Acute phase	17	0.39 ± 0.45	2.5 ^f	65
Convalescent phase	17	0.45 ± 0.49	2.8 ^g	71
Unpaired convalescent phase	15	0.43 ± 0.44	2.7 ^h	53
Healthy adults				
Dutch military	20	0.85 ± 0.37	7.1 ⁱ	100
U.S. laboratory personnel	23	0.63 ± 0.43	4.3 ^j	87

^a One unit is equal to ~1 µg of IgM anti-MenB PS antibody compared with the antigen-binding activity of human anti-MenB PS IgM 5E1 MAb. For calculation of geometric mean values, only the inhibitable binding signal was used, and concentrations of <1.0 unit/ml were assigned a value of 0.5. The superscripts *c* to *j* indicate probabilities, as follows: *c* versus *d* or *e*, *P* < 0.005; *c* versus *f*, *P* > 0.15; *f* versus *g* or *h*, *P* > 0.5; *f* versus *i*, *P* < 0.005; *i* versus *j*, *P* = 0.09.

^b A concentration of 2 units of IgM anti-MenB PS antibody per ml was chosen as a convenient threshold for analysis since sera with antibody concentrations of 2 units/ml or higher gave a signal in the ELISA >2 standard deviations above that of the low-positive control serum sample used in the assay.

from whom paired serum samples were obtained. In this group, the sera of 23 subjects (62%) showed twofold or greater increases in IgM antibody concentration. Of interest, among the patients whose sera showed twofold or greater increases, the geometric mean IgM antibody concentrations in acute- and convalescent-phase sera were 2.1 and 10.4 units/ml, respectively, compared with 11.3 and 10.8 units/ml, respectively, among patients who showed less than twofold increases. Thus, the major difference between responders and nonresponders was higher acute-phase antibody concentrations in the nonresponder group.

IgM anti-MenB PS antibody concentrations in sera from 32

control patients from The Netherlands with culture-proven meningococcal disease caused by serogroup A or C organisms were also measured. The geometric mean IgM anti-MenB PS antibody concentration in acute-phase sera from these patients was 2.5 units/ml, and there was no significant difference in the antibody activity in the respective 17 paired convalescent-phase serum samples (geometric mean, 2.8 units/ml; *P* > 0.5) or the 15 unpaired convalescent-phase serum samples (2.7 units/ml). Only 3 of 32 (10%) convalescent-phase serum samples from the control patients with MenA or MenC disease had IgM anti-MenB PS antibody activity levels of >10 units/ml, and none had antibody activity levels of >25 units/ml (*P* <

FIG. 3. IgG anti-MenB PS antibody-binding titration curves of representative acute-phase (dashed lines) or convalescent-phase (solid lines) sera from two patients with MenB disease. Serial dilutions of sera were added to replicate wells that contained either diluting buffer alone (●) or buffer containing soluble MenB PS (○). After incubation overnight at 4°C, human IgG was detected by alkaline phosphatase-conjugated murine anti-human IgG MAb HP6043. No significant inhibition of antibody binding was observed with the acute-phase serum from patient 212 or the acute- or convalescent-phase sera from patient 211. The titers assigned to these sera were <1:50. (A) Patient 175; (B) patient 212; (C) patient 211.

FIG. 4. Serum antibody responses to MenB PS in patients with invasive MenB disease. Data are for patients from whom paired acute- and convalescent-phase serum samples were obtained ($n = 37$ for IgM [A and B]; $n = 35$ for IgG [C and D]). For clarity, data are stratified on the basis of a less than twofold increase (A and C) and a twofold or greater increase (B and D) in the serum antibody concentration. The indicated antibody responses were calculated by using only the inhibitable binding signal in an ELISA.

0.001 by chi-square analysis [2 degrees of freedom] compared with the respective values for the 94 convalescent-phase serum samples from patients with MenB disease).

The healthy military recruits from The Netherlands had a higher geometric mean IgM anti-MenB PS antibody concentration than the healthy U.S. laboratory personnel (7.1 versus 4.3 units/ml; $P = 0.09$). The military recruits also had twofold higher antibody concentrations than those in acute-phase samples from the patients with MenA or MenC disease ($P < 0.01$; Table 3). Among the military recruits, the proportions of samples with IgM anti-MenB PS antibody concentrations of >2 , >10 , and >25 units/ml were 100, 40, and 5%, respectively; for the 23 U.S. laboratory personnel, the proportions were 87, 22, and 0%, respectively, and for the convalescent-phase sera from patients with MenA or MenC disease the proportions were 63, 9, and 0%, respectively.

IgG anti-MenB PS antibody titers were measured in sera from 92 of the 94 patients with MenB disease. The samples omitted had insufficient volumes. Among the paired samples ($n = 35$), the geometric mean IgG anti-MenB PS antibody titers increased from 1:27 in acute-phase sera to 1:36 in convalescent-phase sera (Table 4; $P < 0.05$). The corresponding geometric mean fold increase was 1.3. The range of fold increases was 0.2 to 29.6. Among the 57 convalescent-phase serum samples from patients for whom acute-phase samples

were lacking, the geometric mean titer was 1:36 (Table 4). Figure 4C and D shows the IgG antibody concentrations in acute- and convalescent-phase sera, respectively, for the 35 patients from whom paired sera were available. Sera from only 7 of the 35 patients (20%) showed twofold or greater increases in antibody titer. In contrast to the IgM responses, when the patients were stratified on the basis of whether they showed twofold or greater or less than twofold increases in IgG anti-MenB PS antibody titers, there was no significant difference in the acute-phase IgG geometric mean antibody titers (26.3 and 29.7 units of IgG antibody per ml, respectively; $P > 0.5$). Among the healthy adults, detectable serum IgG anti-MenB PS antibody was infrequent (10 and 13% of Dutch military recruits and U.S. laboratory personnel, respectively). The IgG titers in sera from the five positive subjects were 1:58, 1:100, 1:79, 1:85, and 1:120.

Effect of age on antibody response. The IgM anti-MenB PS antibody concentrations in convalescent-phase sera from patients 3 to 9 years old were approximately twofold lower than the concentrations in sera from patients 20 to 73 years old (geometric means, 7.9 and 15.7 units/ml, respectively; $P < 0.05$). There also was a trend for lower IgG titers for patients 3 to 9 years old compared with those for patients 20 to 73 years old (geometric mean titers, 1:28 and 1:41, respectively; $P = 0.08$). However, high serum antibody concentrations (>25

units of IgM anti-MenB PS antibody per ml or titers of IgG anti-MenB PS antibody of $\geq 1:100$) were found in sera from patients in the youngest age groups. Overall, there was only a weak relationship between age and the magnitude of the logarithms of the antibody concentrations in convalescent-phase sera (for IgM, $r^2 = 0.06$ and $P < 0.05$; for IgG, $r^2 = 0.08$ and $P < 0.01$).

DISCUSSION

MenB disease remains an important bacterial disease against which no highly effective vaccine is available (for a review, see reference 14). Chemically modified versions of MenB PS have been produced with the ultimate goal of using the modified PSs as components of conjugate vaccines that can induce antibodies that recognize bacterial epitopes, but not self-antigens (e.g., host polysialic acid) (27, 28). For evaluation of the immunogenicities of these vaccines, a sensitive and specific assay is required to measure antibodies of different isotypes and avidities against MenB PS-polysialic acid epitopes.

The ELISA used in the present study was adapted from that described previously by Sutton et al. (50). Important differences in the method used for the present study include the use of an antigen inhibition step for discriminating between specific and nonspecific binding of anti-MenB PS antibodies and, in the IgM assay, inclusion of a standard curve obtained by using a human IgM anti-MenB PS MAb of known concentration. Test sera were also incubated with the antigen at 4°C instead of at room temperature, which increased the sensitivity of the present assay (3, 36).

Our studies indicate that the antibody-binding activity in many patient serum samples is only partially inhibited by soluble MenB PS. With respect to the solid-phase antigen used, lack of inhibition of antibody binding was observed with three different preparations of biotinylated MenB PS, as well as with biotinylated *E. coli* K1 PS, provided by Rachel Schneerson, National Institutes of Health (50). Furthermore, we observed (35a) even higher uninhibitable background binding when assaying MenB PS-reactive MAbs or serum antibodies by an ELISA with native MenB PS noncovalently mixed with methylated human serum albumin as the solid-phase antigen, which was performed by the procedure of Carlone et al. (9). Thus, we do not believe that the problem of nonspecific binding is unique to assays that use biotinylated MenB PS antigen bound to avidin.

The source of uninhibited binding activity in the ELISA is unknown. Possibilities include the presence of antibody specific for MenB PS epitopes that are unique to plate-bound native antigen or to neodeterminants resulting from derivatization or biotinylation of MenB PS. Importantly, membrane-associated MenB PS, when presented on whole bacteria or MenB PS complexed to alum, did not inhibit the nonspecific antibody binding any better than did soluble MenB PS (Fig. 1 and Table 2). These controls were used because of the possibility that the tertiary structure of the PS expressed on the encapsulated bacteria or on PS complexed to alum might differ from that of soluble MenB PS and that bound PS might therefore be a more efficient inhibitor of anti-MenB PS antibodies induced by infection. Indeed, Leinonen and Frasch (33) reported that alum-bound MenB PS inhibited IgG and IgM anti-MenB PS antibodies two- to threefold better than did soluble MenB PS. However, even with the use of MenB PS complexed with alum, those investigators reported that an average of 30% of the IgM or IgG antibody-binding activity was not inhibited. This result suggests that nonspecific binding also was present in their assay. In the present study, the binding

activities of all of the human MenB PS-reactive MAbs or paraproteins were inhibited >90% by either soluble MenB PS or MenB PS-encapsulated bacteria. Taken together, the data suggest that noninhibitable serum antibody binding represents nonspecificity in the ELISA. Therefore, failure to correct for the noninhibitable fraction of antibody-binding activity may result in overestimation of the true serum anti-MenB PS antibody concentrations (Table 2).

The most important conclusions of the present study are that the principal anti-MenB PS antibody response of patients with MenB disease is IgM, although some patients also show IgG responses. Leinonen and Frasch (33), studying a much smaller group of patients, reported similar predominantly IgM anti-MenB PS responses. In our study, children as young as 3 years of age showed high IgM anticapsular antibody responses. The absence of a marked effect of age on the magnitude of the anti-MenB PS antibody response is surprising when contrasted to that observed in patients infected with other PS-encapsulated organisms, such as *H. influenzae* type b (37, 38). However, the youngest age group examined in the present study was 3 to 5 years. Perhaps if sera from patients younger than 3 years had been evaluated, a more pronounced age-related pattern of response would have been apparent.

Another finding of our study was the high prevalence of IgM anti-MenB PS antibody in the serum of healthy adults (87 to 100%), which is consistent with previous reports (16, 30, 33, 36, 54, 55). The reason for the observed trend toward higher IgM antibody concentrations in the Dutch personnel compared with those in the U.S. laboratory personnel is unknown. The difference may reflect more recent exposure to MenB colonization in the Dutch subjects. In contrast to the high prevalence of IgM antibodies, the prevalence of IgG anti-MenB PS antibodies was low, approximately 10 to 15% of the healthy adults (Table 4). The actual prevalence of serum IgG anti-MenB PS antibodies in healthy individuals is controversial. The answer to this question is of practical importance since if serum IgG anti-MenB PS antibodies are highly prevalent in the population, it would imply that most fetuses are exposed to transplacental antipolysialic autoantibodies without suffering harmful effects. However, consistent with our data, Zollinger et al. (54) and Leinonen and Frasch (33) reported very low or undetectable levels of IgG anti-MenB PS antibodies in the sera of healthy adults. In contrast, Devi et al. (12) reported high IgG anti-MenB PS antibody concentrations in most maternal and cord sera tested. The latter investigators used an ELISA that used biotinylated MenB PS as the test antigen. However, they did not use a PS inhibition step to control for the nonspecificity of antibody binding. Given the poor MenB PS inhibition results reported herein (Fig. 1 and 3 and Table 2), the specificity of the IgG anti-MenB PS antibody binding measured by Devi et al. (12) requires reexamination.

The relationship between serum anti-MenB PS antibody and protection against MenB PS disease is controversial. The presence of serum anticapsular antibodies that activate complement-mediated bactericidal activity against serogroup A or C encapsulated *N. meningitidis* correlates with protection (17, 18, 21). However, for group B organisms this correlation is less certain since, when tested with human complement, human anti-MenB PS antibodies are reported not to be bactericidal (53). In separate studies, we found that the IgM anti-MenB PS 5E1 MAb, which was used as the reference standard in the present study, elicited complement-mediated bactericidal activity against a panel of MenB strains by using serum from an agammaglobulinemic subject as a complement source (35). The concentration of the IgM 5E1 MAb required to elicit bacteriolysis ranged from 1 to 2 units/ml. Therefore, it is of

TABLE 4. IgG anti-MenB PS antibody responses of patients recovering from invasive meningococcal disease

Patient group	No. of samples	1/log ₁₀ titer (mean $\pm$ SD)	1/geometric mean titer ^a	% Sera with titer $\geq$ 1:50
Serogroup B disease				
Paired samples				
Acute phase	35	1.43 $\pm$ 0.14	27.0 ^b	6
Convalescent phase	35	1.55 $\pm$ 0.36	35.9 ^c	20
Unpaired convalescent phase	57	1.56 $\pm$ 0.30	36.4 ^d	26
Serogroup A or C disease				
Paired samples				
Acute phase	17	1.47 $\pm$ 0.17	29.8 ^e	18
Convalescent phase	17	1.48 $\pm$ 0.19	30.5 ^f	18
Unpaired convalescent phase	12	1.45 $\pm$ 0.19	28.3 ^g	8
Healthy adults				
Dutch military	20	1.45 $\pm$ 0.17	27.9 ^h	10
U.S. laboratory personnel	23	1.47 $\pm$ 0.20	29.7 ⁱ	13

^a Titers are the reciprocal dilutions of sera that gave an inhibitable signal of an OD of 0.2 (see Materials and Methods). Sera with titers of $<1:50$ were assigned a value of 1:25 for calculation of geometric mean titers. The superscripts *b* to *i* indicate probabilities, as follows: *b* versus *c* or *d*, $P < 0.05$; *e* versus *f* or *g* or *h*, $P > 0.5$; *b* versus *e* or *h* or *i*, $P > 0.3$.

interest that in the present study, two-thirds of the patients with MenB disease had acute-phase IgM anti-MenB PS antibody concentrations of >2 units/ml (Table 3) and nearly one-third had concentrations of >10 units/ml. Thus, a substantial number of these patients developed MenB disease, despite the presence of the high concentrations of IgM anti-MenB PS antibody in acute-phase sera. In addition to the possible lack of protection by the antibodies, several other interpretations of these data are possible. First, IgM anti-MenB PS antibody responses to infection may occur rapidly, and the antibody concentrations found in acute-phase serum may not be representative of those present in serum at the onset of invasive disease. Second, in some patients, despite the presence of protective serum anti-MenB PS antibody concentrations, inhibitors of bacteriolysis such as IgA blocking antibody may also be present (22). Third, in other studies we found that the functional activity of individual human anti-MenB PS IgM MAbs ranged from highly active to minimally active with respect to their ability to elicit complement-mediated bacteriolysis of MenB strains (35) or to confer protection against *E. coli* K1 bacteremia on rats (3). Thus, not all anti-MenB PS antibodies are equally likely to confer protection. Conceivably, the anti-MenB PS antibodies present in the acute-phase sera of some of the patients may have failed to protect against disease because they were representative of the subset of antibodies with poor intrinsic functional activity.

If the protective role of serum IgM anti-MenB PS antibodies is difficult to define, the protective role of serum IgG anti-MenB PS antibody is even more difficult to determine (40). For example, a chimeric IgG anti-MenB PS MAb with variable regions identical to those of the IgM 5E1 MAb bound to MenB PS with much lower avidity than the IgM antibody and required, on a weight basis, greater than 1,000-fold more antibody to elicit opsonization or to confer animal protection against *E. coli* K1 disease (40, 41). Whether all serum IgG anti-MenB PS antibodies show poor functional activity similar

to that of this chimeric IgG antibody is unknown. However, because IgG anti-MenB PS antibodies appear to have much lower avidities than the corresponding IgM antibodies, it is likely that the IgG antibodies play a lesser role in protection against MenB disease.

In summary, our data indicate that MenB PS is immunogenic in most patients recovering from MenB disease, eliciting both serum IgM and serum IgG antibody responses. Most healthy adults also have serum IgM antibody to MenB PS, but IgG antibody reactive with this PS is rarely detected. Finally, the ELISA described herein should be useful for assessing antibody responses to new investigational MenB PS-protein conjugate vaccines and for furthering our understanding of the role of serum anti-MenB PS antibodies in protection against MenB or *E. coli* K1 disease.

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