Measurement of Antibodies against Meningococcal Capsular Polysaccharides B and C in Enzyme-Linked Immunosorbent Assays: Towards an Improved Surveillance of Meningococcal Disease

JESPER ANDERSEN,* LENE BERTHELSEN, AND INGA LIND

Neisseria Department, Statens Serum Institut, DK-2300 Copenhagen S., Denmark

In order to improve the surveillance of serogroup B and C meningococcal diseases, enzyme-linked immunosorbent assays (ELISAs) specific for anti-B immunoglobulin M (IgM) and anti-C IgM and IgG antibodies were developed. The tests were evaluated by using paired sera from 122 patients with and 101 patients without laboratory evidence of meningococcal disease. Fifty-three of 67 patients (79%) with culture-confirmed serogroup B disease had an anti-B IgM antibody response; anti-B IgM levels waned rapidly in children ≤4 years of age. Twenty-four of 25 patients (96%) with culture-confirmed serogroup C disease had an anti-C IgM and/or IgG antibody response (IgM, 92%; IgG, 68%). In patients without evidence of meningococcal disease, 19% of children ≤4 years of age and 69% of those >4 years of age had intermediate anti-B IgM titers. In contrast, only 1 and 5% of these patients had intermediate titers of anti-C IgM and anti-C IgG, respectively. The ELISAs were shown to be powerful tools for discriminating between serogroup B and C diseases in 96 to 100% of culture-confirmed cases. For 90% of patients with culture-negative meningococcal disease, a serogroup-specific diagnosis could be established by examination of paired sera in the ELISAs. As serogroup B and C meningococci account for practically all cases of meningococcal disease in industrialized countries, the availability of these tests may improve surveillance and prevention.

Meningitis and septicemia due to *Neisseria meningitidis* remain a major health problem worldwide (24). Serogroup B and C meningococci account for practically all cases of meningococcal disease in Europe (5) and North America (15).

Since 1986, Denmark has had a high annual incidence of meningococcal disease compared with most other European countries, and in Denmark, approximately 80% of the notified cases were confirmed by culture of *N. meningitidis* (13, 23). Preadmission antibiotic treatment of patients suspected of meningococcal disease, which is recommended by health authorities in several countries, remains a matter of debate (2, 26). In cases of preadmission treatment, noncultural diagnostic methods, such as PCR, antigen detection, and specific antibody determination, become important tools in achieving a diagnosis; during the last 5 to 10 years, an increasing proportion of the culture-negative cases in Denmark have been confirmed by a meningococcal antibody test (MAT) (27). Antibodies towards the capsular polysaccharides are not detected in the MAT; a serological test capable of identifying serogroup B- and C-specific antibodies would improve epidemiological surveillance.

Although the purified B polysaccharide seems to be weakly immunogenic in humans and the C polysaccharide vaccine is effective only in children ≥2 years of age (21), specific immune responses towards the respective polysaccharides have been observed in the majority of adults and 30% of children recovering from serogroup B meningococcal disease (12) and in the majority of patients recovering from serogroup C disease (12, 16). Whether it is possible to establish a definitive serogroup-specific diagnosis and thus discriminate between serogroup B

and C meningococcal diseases on the basis of the presence of specific anticapsular antibodies has been elucidated only to a limited extent (14).

The aim of this study was to develop assays based on the enzyme-linked immunosorbent assay (ELISA) principle for detection of immunoglobulin M (IgM) and IgG antibodies against the meningococcal capsular polysaccharides B and C and to evaluate the assays by testing paired sera from patients with and without meningococcal disease. In preliminary experiments, the anti-B IgG assay did not detect any antibodies in sera from patients recovering from serogroup B meningococcal disease; therefore, this assay was not further developed.

MATERIALS AND METHODS

Study populations. One hundred twenty-two patients with and 101 patients without laboratory evidence of meningococcal disease registered from 1989 to 1994 in the Neisseria Department, Statens Serum Institut, were included. This department serves as a national reference center for the microbiological surveillance of meningococcal disease in Denmark. The sera analyzed were from hospitalized patients whose symptoms were indicative of meningococcal disease. All sera ($n = 456$) investigated were from patients selected according to the criteria described below, and from each patient at least 2 serum samples were available. The materials were divided into three groups. Patients with recent histories of meningococcal disease were assumed not to be included in group I.

(i) Group I: sera from patients without evidence of meningococcal disease. Patients ($n = 101$) in group I had two MAT-negative serum samples taken at the optimal time for demonstration of seroconversion; the first sample was taken at the onset of disease, whereas the second sample was collected within 5 to 21 days after the first one. The first serum sample was negative for meningococcal capsular polysaccharide antigens (A, B, and C) when tested by counterimmunoelectrophoresis. None of the patients were registered through the clinical notification system for communicable diseases or through the laboratory surveillance system (23). The median age of the patients was 9 years (range, 3 months to 84 years), with an age distribution similar to that of patients in whom meningococcal disease was confirmed by culture (group II).

(ii) Group II: sera from patients with culture-confirmed meningococcal disease. Patients ($n = 92$) in group II had meningococcal disease confirmed by culture of *N. meningitidis*. Sixty-seven patients had serogroup B disease, and 25 had serogroup C disease. The serum samples were collected between 0 and 51

* Corresponding author. Mailing address: Neisseria Department, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S., Denmark. Phone: 45 32683475. Fax: 45 32683862.

TABLE 1. Days after onset of disease[a] at which the first and subsequent blood samples were drawn from 92 patients with culture-confirmed meningococcal disease

Sample no.	No. of samples collected on days (% positive by the MAT):						
	0–2	3–4	5–7	8–14	15–21	22–28	29–51
1	71 (14)	13 (31)	4 (100)	3 (67)	1 (100)		
2 or 3	1 (0)	4 (50)	15 (87)	47 (85)	18 (94)	9 (56)	6 (100)

[a] Day of first sample from which *N. meningitidis* was cultured.

days after the collection of the specimen from which *N. meningitidis* was isolated; 91% had the first sample taken between days 0 and 4, while 80% of the subsequent samples were collected between days 5 and 21. All sera were tested by the MAT (Table 1). The median time between two samples was 12 days. The median age of the patients was 13 years (range, 3 months to 69 years).

(iii) Group III: sera from patients with a clinical diagnosis of meningococcal disease confirmed by the MAT. Patients ($n = 30$) in group III had a clinical diagnosis that was not confirmed by culture of *N. meningitidis* but was confirmed by seroconversion or a change in titer of $\geq$3 degrees of potency (DP) by the MAT (see below). The second sample was collected within 7 to 30 days of the first one, which by definition was taken on day 0, i.e., at the onset of the disease. The median age of the patients was 5 years (range, 4 months to 71 years).

ELISAs. An assay for detection of IgM antibodies against the B polysaccharide (anti-B IgM) and two assays for detection of IgG and IgM antibodies against the C polysaccharide (anti-C IgG and anti-C IgM, respectively) were developed.

Control sera. (i) Anti-B IgM assay. The in-house standard reference (SR) serum was a pool of six sera from seropositive patients recovering from culture-confirmed serogroup B meningococcal disease. No international standard for anti-B IgM antibodies is available. The in-house quality control (QC) serum was a pool of three sera with three levels of anti-B IgM antibodies from patients recovering from culture-confirmed serogroup B meningococcal disease. The negative control serum was from a patient with no evidence of meningococcal disease, as described above for group I patients.

(ii) Anti-C IgM assay. The in-house SR serum was a pool of six sera from seropositive patients recovering from culture-confirmed serogroup C meningococcal disease. The in-house SR serum had an anti-C IgM antibody concentration of 43 µg/ml when compared to SR serum 1992 (kindly provided by George M. Carlone, Centers for Disease Control and Prevention [CDC], Atlanta, Ga.). The in-house QC serum pool was a pool of three sera with three levels of anti-C IgM antibodies from patients recovering from culture-confirmed serogroup C meningococcal disease. The negative control serum was from a patient without evidence of meningococcal disease, as described above for group I patients.

(iii) Anti-C IgG assay. The in-house SR serum was from a patient who had a high anti-C IgG antibody titer after vaccination with the A/C meningococcal polysaccharide vaccine. The in-house SR serum had an anti-C IgG antibody concentration of 126 µg/ml when compared to SR serum 1992 (CDC). The in-house QC serum was a pool of three sera with three levels of anti-C IgG antibodies from patients recovering from culture-confirmed serogroup C meningococcal disease. The negative control serum was from a patient without evidence of meningococcal disease, as described above for group I patients.

Each of the three SR sera was assigned a content of 1,000 arbitrary units (AU)/ml of either IgG or IgM antibodies against the B or C polysaccharide.

Methodology. The method developed was modified and optimized from those of Leinonen and Frasch (19) and Salih et al. (22) by the following procedures.

(i) Coating. Microtiter plates (catalog no. 269620; Nunc) were precoated with 100 µl of 2 µg/ml poly-L-lysine (Sigma) (molecular weight, 271,000) per well in carbonate buffer (pH 9.6). The plates were incubated for 2 h at 37°C and washed three times (200 µl per well with contact time of 1 min per step) with phosphate-buffered saline (PBS) (pH 7.4) with a Nunc immunowasher. Plates were subsequently coated with 100 µl of 1 µg/ml serogroup B (kindly provided by Pasteur-Meriéux) or C (Connaught) meningococcal polysaccharide per well in PBS (pH 7.4). For a control, parallel rows were coated with 100 µl of PBS (pH 7.4) per well without polysaccharide antigen. Plates were incubated overnight at 4°C and washed with PBS (pH 7.4) as described above.

(ii) Blocking. For blocking, 150 µl of PBS (pH 7.4) containing Tween 20 (0.1% [vol/vol]) and skim milk (0.5% [wt/vol]) (IRMA) was added to each well. Plates were incubated for 1 h at room temperature and washed with PBS (pH 7.4) containing Tween 20 (0.1% [vol/vol]) as described above.

(iii) Serum samples. All test sera were analyzed for the presence of anti-B IgM, anti-C IgM, and anti-C IgG antibodies. Test sera and the negative control serum were diluted 1:100 in blocking buffer, and 100 µl was added to each of two wells containing antigen (polysaccharide and poly-L-lysine) and to each of two wells containing poly-L-lysine. The appropriate SR and QC sera were diluted 1:200 in blocking buffer, and twofold titrations (six and four times, respectively) with 100 µl per well in antigen wells and in poly-L-lysine wells were made. As a background control, blocking buffer was added to duplicate wells with antigen

and poly-L-lysine. Plates were incubated for 1 h at room temperature and washed with PBS (pH 7.4) containing Tween 20 (0.1% [vol/vol]) as described above.

(iv) Conjugate. A conjugate of rabbit anti-human immunoglobulin labelled with horseradish peroxidase (DAKO) was diluted in blocking buffer (anti-IgG, 1:4,000; anti-IgM, 1:1,000), and 100 µl was added to each well for IgG and IgM assays. Plates were incubated for 1 h at room temperature and washed with PBS (pH 7.4) containing Tween 20 (0.1% [vol/vol]) as described above.

(v) Substrate. *ortho*-Phenylenediamine (catalog no. 4120; Kem-Entec) in citric acid buffer (pH 5.0) (final *ortho*-phenylenediamine concentration, 0.33 mg/ml) was employed as the substrate. The solution was prepared 10 min before use and protected from light. Just before use, a 30% H_2O_2 solution was added to a final H_2O_2 concentration of 0.0125% (vol/vol), 100 µl of the solution was added to each well, and plates were incubated for 10 min at room temperature on a plate rotator table. The reaction was stopped by adding 150 µl of 1 M H_2SO_4 per well.

(vi) Reading. Absorbance values were read at a wavelength of 490 nm, with 630 nm as the reference, in a BIO-TEK EL 312 immunoreader. Each test result was determined as the difference between the optical density (OD) in the antigen well and that found in the corresponding poly-L-lysine well and recorded as the mean of duplicate determinations. The OD test result of each serum sample was transformed to AU per milliliter by the linear portion of the SR curve (minimum, four points) based on a log/log scale. Calculations were performed by the software program Kineticalc, and the assay was accepted when the transformed OD values of the titrated QC serum were within the 95% confidence interval (CI) of the mean antibody concentration of the QC serum, as determined on the basis of the interassay variation during 10 weeks (see Fig. 1), and when the background OD values (in general, <0.020) of the antigen and poly-L-lysine wells were identical. The lower antibody detection limit in each assay was 15.6 AU/ml.

Inhibition ELISAs. Competitive inhibition by the B and C polysaccharide antigens used in the ELISAs was assessed by using a single dilution (1:200) in blocking buffer of each of the anti-B and anti-C polysaccharide SR sera. Each serum dilution was distributed into 10 aliquots. The B and C polysaccharide antigens were dissolved in blocking buffer (100 µg/ml) and titrated twofold nine times. An equal volume of each of the serially diluted polysaccharide solutions was added to nine of the homologous serum aliquots (final polysaccharide concentrations ranged from 50 to 0.2 µg/ml). To the tenth serum aliquot, the same volume of blocking buffer without polysaccharide was added as a control. Other control wells contained equal volumes of polysaccharide (50 µg/ml) and blocking buffer without antiserum. All mixtures were allowed to react for 1 h at 37°C, and the antibody activity of the mixture was then determined by the ELISA procedure described above.

MAT. The MAT is a complement fixation test that is performed routinely in our department (27). The antigen employed in the MAT was a pool of heat-killed (56°C; 20 min) whole cells of serogroup B and C meningococcal strains, including current outbreak strains. Guinea pig serum was used as a complement source. The amount of antibody in a patient's serum sample is traditionally expressed in DP (18). The initial serum dilution was 1:12. One DP corresponds to the amount of antibody resulting in 60% inhibition of hemolysis of the indicator system.

The titers in the MAT were interpreted as follows: 1 to 2 DP, weakly positive; 3 to 5 DP, moderately positive; and $\geq$6 DP, strongly positive. A difference of $\geq$3 DP between two sera was considered to be a significant change in antibody titer.

Statistics. For the comparison of antibody titers determined in both the MAT and ELISA, nonparametric methods were used, as these were not normally distributed. In the analysis of individual changes in antibody titers from one time point to another, the Wilcoxon signed rank sum test was employed. For the comparison of antibody titers of two or more independent groups, the Mann-Whitney two-sample test (Kruskall-Wallis one-way analysis of variance for more than two groups) was used.

A probability level of less than 5% ($P < 0.05$) was considered to be significant. The EPI-INFO software produced by CDC was used in this analysis.

RESULTS

Variations in ELISAs. The intra- and interassay variations were estimated on the basis of repeated determinations of the mean antibody titers of the QC sera used in the assays. For all three assays, the general intraassay coefficient of variation (CV) was found to be below 10% for determinations in duplicate. On the basis of four determinations per week for 10 weeks, the interassay CV within 1 week was estimated to be below 20% in each of the three assays: anti-B IgM CV, 3 to 13%; anti-C IgG CV, 3 to 19%; and anti-C IgM CV, 1 to 12% (Fig. 1). The mean antibody titer obtained by four weekly determinations was very constant through 10 weeks for each QC serum (Fig. 1). A difference of at least 1.5-fold in the antibody titers of two serum samples was significant.

Inhibition ELISAs. The binding of IgM antibodies in the SR serum to the B polysaccharide bound in the ELISA plate was
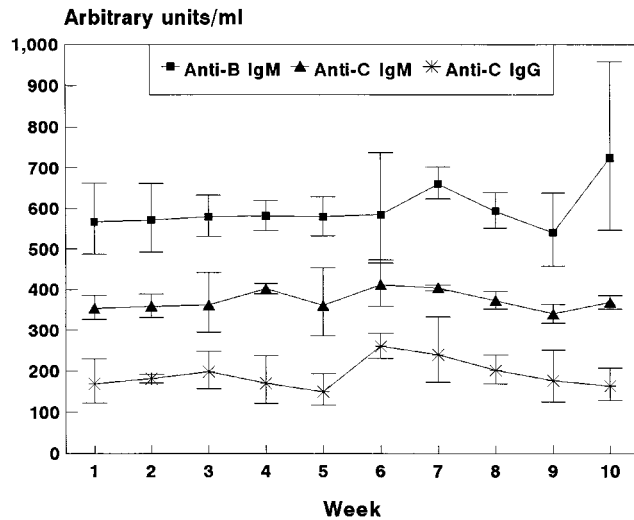
FIG. 1. Mean antibody concentrations of anti-B IgM, anti-C IgM, and anti-C IgG in three QC sera in ELISAs during 10 weeks. Mean concentrations (in AU/milliliter) were based on four assays per week (error bars, 95% CI = 2 standard deviations).

inhibited by >99% when free B polysaccharide at a concentration of 50 µg/ml was present in the solution (Fig. 2). Likewise, a concentration of 50 µg of free C polysaccharide per ml in the solution was able to inhibit the binding of IgM and IgG antibodies in the homologous SR sera by >99 and >96%, respectively. However, to achieve 50% inhibition of binding to the bound homologous polysaccharide, only 0.2 µg of free C polysaccharide per ml and about 2 µg of free B polysaccharide per ml were needed in the solution (Fig. 2).

Group I: sera from patients without evidence of meningococcal disease. (i) MAT. All 101 patients included in group I were seronegative (see Materials and Methods).

(ii) ELISA. There was no difference between the individual anticapsular antibody levels of the first (acute-phase) and second serum samples from any of these patients. The results
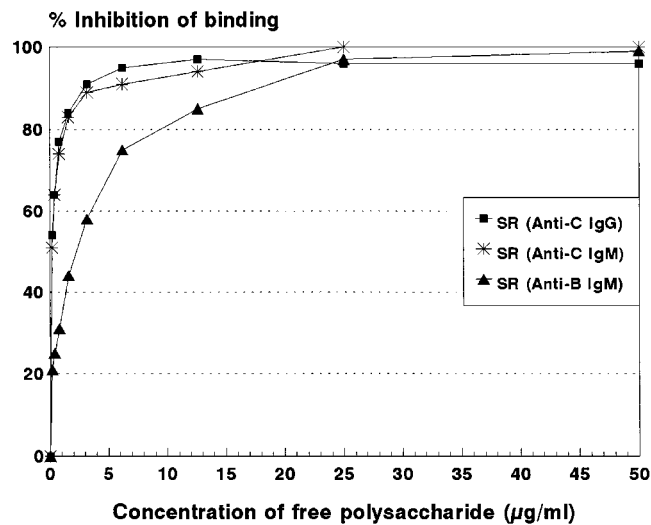

FIG. 2. Percent inhibition of binding in ELISAs of specific antibodies in three SR sera to meningococcal B or C polysaccharide by homologous antigen.

obtained by testing the acute-phase sera were used in this analysis.

Anti-B IgM antibodies above the lower detection limit (>15.6 AU/ml) were observed in 55% of the patients. The distribution of anti-B IgM antibody levels in patients ≤4 years of age differed significantly ($P < 0.001$) from that observed in patients of >4 years, as only 19% of the younger patients compared with 72% of the older patients had detectable anti-B IgM antibodies (Table 2). Among the 101 patients without evidence of meningococcal disease, 99% had an anti-B IgM titer of <150 AU/ml (95% CI, 120 to 190 AU/ml). In contrast, very few of the 101 patients without meningococcal disease had anti-C IgM and anti-C IgG antibodies above the lower detection limit (>15.6 AU/ml); only 2% had anti-C IgM antibodies, whereas 6% had anti-C IgG antibodies (Table 3). No association between the age of the patient and the level of anticapsular antibodies could be demonstrated; 99% of these patients had an anti-C IgM titer of <25 AU/ml (95% CI, 20 to 30 AU/ml) and an anti-C IgG titer of <60 AU/ml (95% CI, 40 to 90 AU/ml).

On the basis of these findings, an anti-B IgM titer of ≥150 AU/ml, an anti-C IgM titer of ≥25 AU/ml, and an anti-C IgG titer of ≥60 AU/ml were considered to be positive test results, whereas titers between the lower detection limit and the positive cutoff value were designated intermediate (Tables 2 and 3).

Group II: sera from patients with culture-confirmed meningococcal disease. (i) MAT. Of the 92 patients, 77% were seronegative in the MAT of the first serum sample. When the first sample was collected within 0 to 4 days, 83% were seronegative, whereas only one of eight patients from whom the first sample was collected after day 4 was seronegative (Table 1). In subsequent sera, the median MAT titer level increased significantly ($P < 0.001$), by 5 DP (range, 0 to 13 DP).

Overall, 80 of the 92 patients (87%) with culture-confirmed meningococcal disease were seropositive in the MAT for at least one sample; 35 (38%) were strongly positive (range, 6 to 13 DP), 24 (26%) were moderately positive (range, 3 to 5 DP), and 21 (23%) were only weakly positive (range, 1 to 2 DP). Twelve (13%) remained seronegative in both samples. A seroconversion or change in titer of ≥3 DP in the MAT was seen in 50 patients (54%), 26 (28%) had a <3-DP change, and 4 (4%) had identical positive test results.

When consecutive samples were taken at optimal times, with the first sample collected in the acute phase (days 0 to 4) and the second one collected within days 5 to 21 after admission, the clinical diagnosis of meningococcal disease was confirmed by a change of ≥3 DP in the MAT for 44 of 72 patients (61%) and 64 were found to be seropositive in at least one of the two samples (diagnostic sensitivity of the MAT, 64 of 72 = 89%).

(ii) ELISA: patients with serogroup B disease. The median anti-B IgM titer of the acute-phase sera did not differ from that observed in sera from group I patients; 6% had a titer of ≥150 AU/ml. Without any association to the age of the patient, a significant anti-B IgM titer increase ($P < 0.001$) was observed in patients recovering from serogroup B meningococcal disease; the median titer increased from <15.6 AU/ml (≤4 years of age) and 28 AU/ml (>4 years of age) in the acute-phase sera to 75 and 142 AU/ml, respectively, between days 5 and 14 (Table 2). The median anti-B IgM titer of the acute-phase sera of these two age groups differed significantly ($P < 0.001$), whereas no difference was observed in sera collected between days 5 and 14 (Table 2). After day 14, 37% of patients >4 years of age had a positive titer of ≥150 AU/ml, whereas all of the nine younger patients were seronegative or had intermediate titers. Seventy-six percent ($n = 51$) had a >1.5-fold change in

TABLE 2. Age-specific medians and distributions of anti-B IgM antibody titers for patients with no evidence of meningococcal disease and patients with serogroup B meningococcal disease

Age (yr)	Parameter (unit)	With no evidence of meningococcal disease (group I)	With serogroup B meningococcal disease (group II)		
			Samples collected days 0–4	Samples collected days 5–14	Samples collected after day 14
>4	No. of patients	69	39	33	11
	Median anti-B IgM titer (AU/ml)	31[a]	28[a]	142	113[a]
	Distribution (%):				
	Negative (<15.6 AU/ml)	28	34	6	9
	Intermediate (16–149 AU/ml)	69	61	45	54
	Positive[b] (≥150 AU/ml)	3	5	49	37
≤4	No. of patients	32	26	17	9
	Median anti-B IgM titer (AU/ml)	<15.6[a]	<15.6[a]	75	<15.6[a]
	Distribution (%):				
	Negative (<15.6 AU/ml)	81	81	12	56
	Intermediate (16–149 AU/ml)	19	12	47	44
	Positive[b] (≥150 AU/ml)	0	7	41	0

[a] $P < 0.05$ between the two age groups.
[b] For definition of cutoff value, see text.

the anti-B IgM titer, and a >4-fold change was seen in 42% ($n = 28$). The overall median titer change upon infection was 2.6-fold (range, 1- to 44-fold). Of the 16 patients without a change in titer, 2 were seropositive (titer of ≥150 AU/ml) in both samples.

In patients with serogroup B disease, a significant change in anti-B IgM titer at the intermediate level or titers of ≥150 AU/ml were demonstrated in 79% (53 of 67). When the two serum samples had been collected in the acute phase and between days 5 and 14, anti-B IgM antibodies were demonstrated in 86% (43 of 50). Among patients with serogroup B disease and with a ≥3-DP seroconversion or change in titer in the MAT, anti-B IgM antibodies could be demonstrated in 33 of 39 (85%).

None of the patients with serogroup B disease had detectable anti-C IgM antibodies. In three patients (>4 years of age), all of whom had an anti-B IgM titer of ≥150 AU/ml, an intermediate anti-C IgG titer (16 to 59 AU/ml) was demonstrated.

(iii) ELISA: patients with serogroup C disease. The median anti-C IgM and anti-C IgG titers in the acute-phase sera from patients with serogroup C disease differed significantly from those found in sera from group I patients; 53% had an anti-C IgM titer of ≥25 AU/ml, and 11% had an anti-C IgG titer of ≥60 AU/ml (Table 3).

As the anti-C IgM antibody titer was already high in the acute-phase sera of half of these patients (Table 3), the median anti-C IgM titer did not increase significantly upon recovery. However, 64% ($n = 16$) showed a >1.5-fold change in IgM titer and 36% ($n = 9$) had a >4-fold change. The overall change in median IgM titer upon infection was 2.1-fold (range, 1- to 42-fold). Among the nine patients without a change in anti-C IgM titer, seven were seropositive (≥25 AU/ml) in paired samples. In patients with serogroup C disease, a significant change in anti-C IgM titer and/or titers of ≥25 AU/ml were demonstrated in 92% (23 of 25). The levels of IgM antibodies waned after day 14 (Table 3). Among those who had a ≥3-DP increase in the MAT, anti-C IgM antibodies could be demonstrated in 100% (11 of 11).

The median anti-C IgG titer increased significantly ($P = 0.002$) upon recovery compared with that in the acute-phase sera (Table 3). Sixty percent ($n = 15$) showed a >1.5-fold change in IgG titer, and 36% ($n = 9$) had a >4-fold change. The overall change in median IgG titer upon infection was

TABLE 3. Medians and distributions of anti-C IgM and IgG antibody titers for patients with no evidence of meningococcal disease and patients with serogroup C meningococcal disease

Class of immunoglobulin	Parameter (unit)	With no evidence of meningococcal disease (group I) ($n = 101$)	With serogroup C meningococcal disease (group II)		
			Samples collected days 0–4 ($n = 19$)	Samples collected days 5–14 ($n = 16$)	Samples collected after day 14 ($n = 13$)
IgM	Median titer (AU/ml)	<15.6	30	157	56
	Distribution (%):				
	Negative (<15.6 AU/ml)	98	47	19	23
	Intermediate (16–24 AU/ml)	1	0	0	8
	Positive[a] (≥25 AU/ml)	1	53	81	69
IgG	Median titer (AU/ml)	<15.6	<15.6	51	65
	Distribution (%):				
	Negative (<15.6 AU/ml)	94	73	25	15
	Intermediate (16–59 AU/ml)	5	16	31	31
	Positive[a] (≥60 AU/ml)	1	11	44	54

[a] For definition of cutoff value, see text.

TABLE 4. Diagnostic values of anti-B IgM, anti-C IgM, and anti-C IgG antibody determinations for patients with culture-confirmed meningococcal disease (group II)

Patients	No. of patients								
	Anti-B IgM antibodies			Anti-C IgM antibodies			Anti-C IgG antibodies		
	Positive[a]	Negative[b]	Total[c]	Positive[d]	Negative[e]	Total[f]	Positive[g]	Negative[h]	Total[i]
Serogroup B disease	27	40	67	0	67	67	2	65	67
Serogroup C disease	1	24	25	23	2	25	12	13	25
Total	28	64	92	23	69	92	14	78	92

[a] ≥150 AU/ml; predictive value of a positive test, 96% (27/28).
[b] Predictive value of a negative test, 38% (24/64).
[c] Sensitivity, 40% (27/67); specificity, 96% (24/25).
[d] ≥25 AU/ml; predictive value of a positive test, 100% (23/23).
[e] Predictive value of a negative test, 97% (67/69).
[f] Sensitivity, 92% (23/25); specificity, 100% (67/67).
[g] ≥60 AU/ml; predictive value of a positive test, 86% (12/14).
[h] Predictive value of a negative test, 83% (65/78).
[i] Sensitivity, 48% (12/25); specificity, 97% (65/67).

2.3-fold (range, 1- to 15-fold). Among the 10 patients without a significant change in anti-C IgG titer, 2 had a titer of ≥60 AU/ml in paired samples. In patients with serogroup C disease, a significant change in anti-C IgG titer or titers of ≥60 AU/ml were demonstrated in 68% (17 of 25). IgG antibodies persisted after day 14 (Table 3). Among those who had a ≥3-DP increase in the MAT, anti-C IgG antibodies could be demonstrated in 91% (10 of 11).

When the results of both the IgM and IgG assays were included, 96% (24 of 25) of the patients with group C disease were seropositive and no association between the anti-C antibody level and age was demonstrated.

Nine of the 18 patients >4 years of age had an anti-B IgM titer change at the intermediate level (16 to 149 AU/ml) (range, 32 to 121 AU/ml), but all of these had an anti-C IgM titer that exceeded the cutoff value (≥25 AU/ml) (range, 27 to 1,350 AU/ml).

(iv) Distinction between serogroup B and C meningococcal diseases by ELISAs. For patients with culture-confirmed meningococcal disease, a serogroup B- or C-specific diagnosis was obtained for 96 to 100% (Table 4). For all three assays, the predictive value of a positive test result was >85%. The predictive value of a negative test result in the anti-C IgM assay was 97%.

Group III: sera from patients with a clinical diagnosis of meningococcal disease confirmed in the MAT. (i) MAT. Three patients (10%) were seropositive (range, 0 to 6 DP) in the acute-phase sample. More than half of the patients had a strongly positive MAT result for the second sample, with a median titer of 7 DP (range, 3 to 15 DP). The median increase in the MAT was 6 DP (range, 3 to 15 DP).

(ii) ELISA. Twenty-seven patients (90%) could be classified as having either serogroup B ($n = 16$) or C ($n = 11$) meningococcal disease. All patients diagnosed as having serogroup B disease had a significant change in anti-B IgM titer (median change, 10.1-fold) (Table 5). In 12 of these patients, an anti-B IgM titer of ≥150 AU/ml was demonstrated. Among the 11 patients diagnosed as having serogroup C disease, 10 had a significant change in anti-C IgM titer (median change, 3.2-fold) and 8 had a significant change in anti-C IgG titer (median change, 2.4-fold) (Table 5).

Four patients, two classified as having serogroup B meningococcal disease and two classified as having serogroup C meningococcal disease, had intermediate titers against the heterologous C and B polysaccharides, respectively.

DISCUSSION

Determinations of antibodies to the meningococcal serogroup A and/or C polysaccharides have mainly been applied in studies of seroepidemiology (22), of patients with invasive diseases (16, 25), or of vaccinees in order to monitor the immunological response upon vaccination (4, 9). Only limited information about the occurrence of antibodies to the serogroup B polysaccharide is available, although several methods for the measurement of anti-B antibodies have been introduced (3, 12, 19, 20). Our objective was to improve the surveillance of meningococcal disease by establishing serological tests that are able to discriminate between diseases caused by serogroup B and C meningococci. For the determination of anti-B and anti-C antibodies, we decided to use the principles of recently described ELISAs. With a view to optimization, we compared

TABLE 5. Median anticapsular antibody titers and fold increases in titer between consecutive samples from patients with MAT-confirmed meningococcal disease (group III)

Patients (n)	Sample no.	Anti-B IgM antibodies		Anti-C IgM antibodies		Anti-C IgG antibodies	
		Titer (AU/ml)	Fold increase (range)	Titer (AU/ml)	Fold increase (range)	Titer (AU/ml)	Fold increase (range)
Serogroup B disease (16)	1	<15.6		<15.6		<15.6	
	2	187	10.1 (2.7–49)[a]	<15.6	1 (1–2.7)	<15.6	1 (1–3.1)
Serogroup C disease (11)	1	<15.6		<15.6		<15.6	
	2	<15.6	1 (1–4.8)	82	3.2 (1–29.1)[b]	39	2.4 (1–12)[c]

[a] All 16 patients had a significant increase in titer.
[b] Of 11 patients, 10 had a significant increase in titer.
[c] Of 11 patients, 8 had a significant increase in titer.

the following three methods of coating microtiter plates: (i) coating with a mixture of methylated human serum albumin and polysaccharide (4), (ii) coating with chemically modified (phenylated) polysaccharide (17), and (iii) coating with polysaccharide after precoating with poly-L-lysine (19) (unpublished results). In our hands, the last procedure with modifications showed reproducible results with intra- and interassay CVs of <10 and 20% (range, 1 to 19%), respectively (Fig. 1). During 1 week only, the anti-C IgG assay had an interassay CV of 19%, which is, however, acceptable. During the 10-week period, the tests were performed by one person only (L.B.), but the reproducibility was maintained at the described level with different lots of reagents and microtiter plates (unpublished results). Some human sera may show nonspecific binding to poly-L-lysine (1, 19), which was demonstrated in sera from patients with as well as without meningococcal disease in the present investigation; some even had high but stable titers to poly-L-lysine in consecutive sera (unpublished results). However, by using the inclusion of poly-L-lysine without meningococcal antigen as a control for each serum sample (22), this problem could be ignored. The antibodies in the appropriate SR serum were demonstrated to be directed against the homologous polysaccharide antigen, as binding was almost completely inhibited by high concentrations of that antigen in free solution (Fig. 2).

The ELISAs were applied to sera from patients with and without evidence of meningococcal disease, and the performances of the tests were further evaluated with sera from patients whose clinical diagnosis was confirmed by an increase of at least threefold ($\geq$3 DP) in MAT titer. In the present study, nearly 90% of the 92 patients with culture-confirmed meningococcal disease became seropositive, as demonstrated by the MAT.

The 101 patients in group I were selected to fulfil the following criteria: (i) a recent history of meningococcal disease should be most unlikely (see Materials and Methods), and (ii) these patients should match the patients with culture-confirmed meningococcal disease (group II) according to age. Thus, sera from these patients were considered to be appropriate in defining the cutoff for a positive test result. Intermediate titers of anti-B IgM were found to be present in more than 50% of these patients. However, only 19% of patients $\leq$4 years of age had anti-B IgM antibodies, whereas these antibodies were present in 69% of patients >4 years of age (Table 2). From the literature, limited data about the presence of anti-B IgM antibodies in healthy individuals are available, but the results are conflicting. Whereas some investigators found that these antibodies were absent from human sera (11), others reported anti-B IgM to be present in most healthy adults (6, 19, 21). Low levels of anti-B IgG may also be present in sera from healthy adults (19) and umbilical cord sera from infants (6). In preliminary experiments, we did not find anti-B IgG antibodies in sera from patients in the convalescent phase of serogroup B meningococcal disease. Our finding is in agreement with previous studies showing that the antibody response upon serogroup B infection is primarily of the IgM class (11, 19).

The presence of intermediate titers of anti-B IgM in patients with no evidence of meningococcal disease remains a controversial issue, as the purified serogroup B polysaccharide is nonimmunogenic in humans (21). These antibodies also react with an identical epitope in the human embryonal brain (8). Anti-C IgM antibodies were present in 2% and anti-C IgG antibodies were present in 6% of sera from patients without evidence of meningococcal disease (Table 3).

Upon infection with serogroup B meningococci, 79% of the patients had an anti-B IgM response and more than 40%

among both children $\leq$4 years of age and those that were older had titers of $\geq$150 AU/ml (Table 2) during the second week of the disease. However, the levels of antibodies waned rapidly in patients $\leq$4 years of age. When consecutive sera were collected at optimum times (on admission and during the second week), 86% of both younger and older patients could be demonstrated to have serogroup B disease, as determined by the presence of anti-B IgM antibodies. A previous study demonstrated that children less frequently had an immune response upon serogroup B infection than did young adults (12).

Anti-C antibodies could be demonstrated in 96% of patients with serogroup C disease, a finding in agreement with previously published results (16). The lack of immunogenicity of the purified C polysaccharide vaccine in children <2 years of age (21) remains controversial. In the present investigation, three of four children <2 years of age had a significant anti-C antibody response upon serogroup C infection. Anti-C IgM antibodies were demonstrated in 92% of the patients, whereas anti-C IgG antibodies were demonstrated in 68%. An anti-C IgM response was detected in all seropositive patients but one, whereby the IgM assay was concluded to be the most useful due to the higher sensitivity. Furthermore, an anti-C IgM titer of $\geq$25 AU/ml was already present in the acute-phase sample of more than half of the patients (Table 3), which indicated that the serogroup C polysaccharide had induced an immune response after pharyngeal acquisition (10), shortly before the development of invasive disease (7).

The serogroup B ($\alpha2\rightarrow8$) and C ($\alpha2\rightarrow9$) polysaccharides are homopolymers of the same polysialic acid, and a cross-reactive epitope is known to exist (28). In a minor proportion of patients with group B disease, low titers of antibodies cross-reacting with the C polysaccharide have previously been demonstrated (3, 16). However, in the present investigation, 50% of the patients >4 years of age with group C disease had intermediate titers (16 to 149 AU/ml) of anti-B IgM. All of these patients, however, had an anti-C IgM titer of $\geq$25 AU/ml. Intermediate anti-B IgM titers were also present in an identical proportion of patients without meningococcal disease (Table 2). Thus, these minor cross-reactions did not cause interpretative problems and each test was highly specific (96 to 100%) in establishing the serogroup-specific diagnosis for patients with meningococcal disease (Tables 4).

In patients with culture-confirmed meningococcal disease, an 85 to 100% correlation between a $\geq$3-DP rise in the MAT and a definitive serogroup-specific diagnosis in ELISA was demonstrated. When the performances of the ELISAs with consecutive sera from culture-negative patients who had the clinical diagnosis confirmed by a $\geq$3-DP rise in the MAT were evaluated, a serogroup-specific diagnosis could be established for 90% of these patients (Table 5). During the follow-up of a Norwegian vaccine trial, a serogroup-specific diagnosis based on anticapsular antibodies was established by ELISA for 59% of 37 culture-negative cases of meningococcal disease (14). Those investigators also used precoating with poly-L-lysine but did not implement subtraction for nonspecific binding. The serogroup-specific diagnosis in that study was based on the presence of anti-B IgM or anti-C IgG, but we have also demonstrated the significance of including anti-C IgM, whereby the diagnostic sensitivity was improved without decreasing the specificity.

In conclusion, the ELISAs described here for the determination of antibodies proved to be powerful tools in discriminating between serogroup B and C meningococcal diseases. The availability of these tests may improve the surveillance of meningococcal disease and thereby the basis on which deci-

sions to implement vaccination in connection with outbreaks of serogroup C disease are made.

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