

## Class-Specific Antibody Response to Group B *Neisseria meningitidis* Capsular Polysaccharide: Use of Polylysine Precoating in an Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay was developed for measuring immunoglobulin class-specific antibodies to *Neisseria meningitidis* capsular polysaccharides. The polysaccharides were fixed to the solid phase by precoating the polystyrene surface with basic polyamino acids. Polylysine precoating was found to give optimal antibody values with the meningococcal polysaccharides studied. The enzyme-linked immunosorbent assay was used to measure antibodies against group B meningococcal polysaccharide in healthy adults and in patients with group B meningitis. Antibodies to group B polysaccharide in sera obtained from healthy adults were primarily immunoglobulin M (IgM). Although the antibody response to disease was mostly IgM, both IgG and IgM antibodies increased. Infection with group B organisms stimulated anti-group B polysaccharide antibodies even in young patients, aged 6 to 7 months, and the magnitude of the B polysaccharide response increased with the age of the patient.

Although the enzyme-linked immunosorbent assay (ELISA) procedure has been commonly used for measuring antibodies to protein antigens, application of ELISA for polysaccharides has not been very successful. Negatively charged polysaccharides do not attach easily to the polystyrene commonly used as the solid phase in ELISA. Antibodies to pneumococcal polysaccharides have been measured by ELISA either by the sandwich method, in which the polystyrene surface is precoated with pneumococcal antibodies (2, 20), or by fixing pneumococcal polysaccharides directly to the polystyrene (17). Pneumococcal polysaccharides have been also covalently bound to polylysine, and these complexes have been fixed to the polystyrene surface (12).

Antibodies against group A and C meningococcal polysaccharides in sera obtained from meningitis patients or from vaccinees have been measured by passive hemagglutination (1, 10) and by radioimmunoassay (3, 11, 19). Studies have shown the group B meningococcal capsular polysaccharide to be a comparatively poor immunogen both in natural infections (13, 25) and in vaccination trials (22). A Farr-type radioimmunoassay was developed that requires intrinsically labeled group B polysaccharide (14, 22), and a solid-phase radioimmunoassay has recently been developed (23).

Our aim was to develop an ELISA for measuring immunoglobulin class-specific antibodies to meningococcal polysaccharides, with special reference to the group B polysaccharide. An ELISA which used polylysine precoating was developed and used to measure the antibody response to the group B polysaccharide in patients with group B meningococcal disease.

### MATERIALS AND METHODS

**Meningococcal polysaccharides.** Capsular polysaccharide antigens of *Neisseria meningitidis* groups A, C, Z, and 29E were isolated by the Cetavlon method of Gotschlich (8). The group B polysaccharide was provided by Connaught Laboratories, Inc., Swiftwater, Pa. The polysaccharides contained less than 1% protein and nucleic acid.

**Serum specimens.** Ten serum samples from healthy adults were used for calibration of the ELISA method. Acute and convalescent sera obtained from patients (aged 6 months to 17 years) with group B meningococcal disease were kindly provided by P. H. Makela, National Public Health Institute, Helsinki, Finland.

**ELISA method for anti-polysaccharide antibodies.** Polystyrene cuvettes (Gilford Instrument Laboratories Inc., Oberlin, Ohio) were precoated by incubation with 300  $\mu$ l of basic polyamino acid solution containing 1 to 10  $\mu$ g of poly-L-arginine, poly-L-histidine, poly-L-lysine, or poly-L-ornithine per ml (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M phosphate-buffered saline (PBS), pH 7.4, for 5 h at 28°C. (This temperature was selected to be slightly above the highest expected ambient temperature.) We found that only those polyamino acids with estimated molecular weights of ap-

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TABLE 1. Comparison of ELISA antibody values of a human serum against five meningococcal polysaccharides with cuvettes precoated with one of four basic polyamino acids

Meningococcal polysaccharide	ELISA units <sup>a</sup> for cuvettes precoated with:			
	Polyarginine	Polylysine	Polyornithine	Polyhistidine
A	19	157	88	2
B	34	37	17	0
C	18	89	36	1
29E	24	31	32	1
Z	23	32	31	0

<sup>a</sup> ELISA units in a 1:200 serum dilution.

proximately 150,000 or greater could be used to precoat the cuvettes. After the cuvettes were washed twice with 600  $\mu$ l of PBS, 300  $\mu$ l of polysaccharide solution (0.5 to 10  $\mu$ g of polysaccharide per ml of PBS) was added, and the cuvettes were incubated overnight at 28°C. The cuvettes were washed seven times with 0.9% NaCl solution containing 0.1% Brij 35 (Sigma) with the Gilford EIA PR-50 instrument (Gilford). Serum samples were diluted (usually 1:200) in PBS containing 0.1% Brij 35 and 0.2% human albumin (PBS-Brij-HSA) and then added to the cuvettes for 4 h at 28°C. After being washed as described above, 300  $\mu$ l of conjugate solution (either alkaline phosphatase-conjugated swine anti-human gamma chain [IgG] or mu chain [IgM] [Orion Diagnostics, Helsinki, Finland] or alkaline phosphatase-conjugated goat anti-human immunoglobulin, prepared as described previously [4] and diluted with PBS-Brij-HSA) was added, and the cuvettes were incubated for 2.5 h at 28°C. The cuvettes were again washed, and 300  $\mu$ l of freshly prepared substrate solution (1 mg of *p*-nitrophenyl phosphate [Sigma no. 104] in 1 ml of 1 N Tris buffer [pH 9.8] containing 0.3 mM MgCl<sub>2</sub>) was added to each cuvette. The cuvettes were left at room temperature for at least 20 min, and then the absorbances were read at 405 nm. After subtracting the background calculated for a cuvette to which PBS-Brij-HSA was added instead of a serum sample, optical density (OD) values for 100 min were calculated, and the results were expressed as ELISA units (OD<sub>100min</sub>  $\times$  100). OD values for the background were usually less than 0.3.

The specificity and sensitivity of the IgG and IgM conjugates were examined by ELISA. Normal serum was fractionated on Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, N.J.), and the fractions were used to sensitize cuvettes for ELISA. Both conjugates were found to be highly specific and to have similar sensitivity.

**ELISA inhibition studies.** Alum-bound B polysaccharide was prepared by a modification of the method of Goldschneider et al. (7). Briefly, 100  $\mu$ l of 0.05 M aluminum hydroxide (alum) was pelleted in a Beckman Microfuge B (Beckman Instruments, Fullerton, Calif.) for 1 min. Next, 100  $\mu$ l of group B polysaccharide solution (1 mg/ml) was mixed with the alum, and after 5 min the mixture was again pelleted. Four serum samples from healthy adults and four from meningitis patients were diluted 1:5 in saline solution, and then 100  $\mu$ l of this solution was added to the alum-bound

polysaccharide or to alum alone. After 2 h at room temperature with occasional mixing, the serum samples were separated from the alum by centrifugation. Additional serum samples from the same individuals were diluted 1:5 and incubated with the same volume of soluble group B polysaccharide (100  $\mu$ g/100  $\mu$ l) at room temperature for 2 h. Group B polysaccharide antibodies were measured by ELISA at a final serum dilution of 1:100 in PBS-Brij-HSA. Anti-human IgG and IgM conjugates were used.

## RESULTS

**Optimal antigen coating of cuvettes.** The optimal concentration for basic polyamino acids and meningococcal capsular polysaccharides were determined by checkerboard titration with anti-human immunoglobulin conjugate. The optimal concentration of basic polyamino acid for pre-coating was 5  $\mu$ g/ml, and the optimal concentration of polysaccharide was 1 to 2  $\mu$ g/ml. Table 1 shows ELISA antibody values for a representative serum against five meningococcal capsular polysaccharides when different basic polyamino acids were used for pre-coating the cuvettes. Precoating with polylysine gave the highest antibody values with the lowest background for the five meningococcal polysaccharides studied. For groups 29E and Z, polylysine and polyornithine gave about the same ELISA antibody values. Precoating with polyhistidine was unsuccessful: 10 serum samples gave background values against all meningococcal polysaccharides studied.

**Correlation between OD values and serum dilutions.** One serum sample from a healthy adult and one serum from a convalescent meningitis patient were serially diluted (1:100 to 1:1,600) and examined with immunoglobulin class-specific conjugates. OD versus serum dilution curves for these sera are shown in Fig. 1. The curves for IgG and IgM antibodies in both sera were parallel, and OD values decreased as a function of dilution.

**Inhibition of antibody binding by group B polysaccharide.** The ability of group B polysaccharide to inhibit the binding of IgG and IgM antibodies in ELISA was studied by absorbing four sera from healthy adults and four sera from convalescent meningitis patients (aged 14 months to 8 years) with the same amounts of either soluble or solid-phase-bound polysaccharide. Table 2 shows the mean ELISA antibody values in these sera before and after absorption with the two polysaccharide preparations. Treatment of patient sera with alum-bound or soluble group B polysaccharide decreased IgG antibody values by 71 and 32%, respectively. The corresponding inhibition for IgM antibodies was 62 and 21%; respectively. In the sera from healthy adults, alum-bound and soluble polysaccharide inhibited 70 and 20%, respectively, of

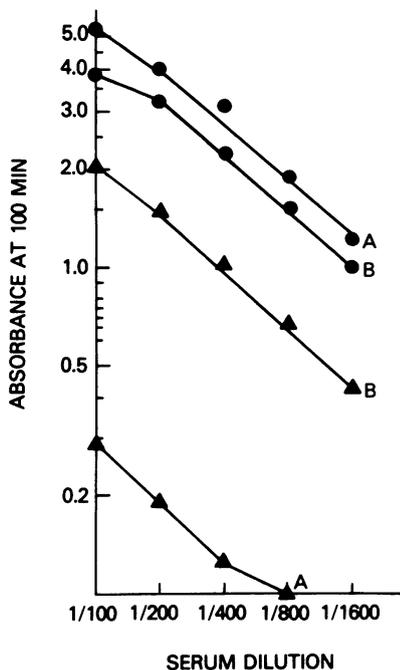


FIG. 1. OD-versus-serum-dilution curves for IgG (▲) and IgM (●) antibodies in two sera, obtained from a healthy adult (A) and from a patient with group B meningococcal meningitis (B).

binding of IgM antibodies. The inhibition of antibody activity with solid-phase-bound group B polysaccharide was thus two to four times more efficient than it was with soluble polysaccharide. Overall IgG antibody levels in healthy adults were very low, and ELISA antibody values decreased to background level.

**Antibodies against group B polysaccharide in healthy adults and meningitis patients.** Acute and convalescent (taken after a 2- to 3-week interval) sera from 10 patients with group B meningococcal meningitis were tested by ELISA with immunoglobulin class-specific conjugates (Fig. 2). IgG and IgM antibodies to group B polysaccharide were detected in all acute sera studied,

although the antibody levels in two small children, 6 and 7 months old, were less than 30 ELISA units. IgM antibodies increased in all 10 patients, and IgG antibodies increased, but to a lower extent, in 8 of 10 patients. The intensity of the response increased with the age of the patient. Antibody responses were predominantly IgM, the fold increase between acute and convalescent sera varying from 2.3 to 9.3. Increases in IgG antibodies were usually less than twofold, although the IgG antibody levels in acute-phase sera, expressed as ELISA units, were usually higher than the corresponding IgM levels.

Only very low amounts of IgG antibodies were found in sera obtained from healthy adults. The mean IgG antibody level was 10 ELISA units whereas the level of IgM antibodies varied from 9 to 171 ELISA units, and only one serum had an IgM antibody level of less than 20 ELISA units.

**DISCUSSION**

Radioimmunoassay, based on Farr's technique, has been used for measuring antibody responses to the group A and C meningococcal polysaccharides (11). Attempts to develop a corresponding Farr assay in which extrinsically labeled polysaccharide is used for detection of group B polysaccharide antibodies have been unsuccessful; however, an intrinsically labeled Farr assay and solid-phase radioimmunoassay have recently been developed for measurement of group B antibodies (22, 23). Antibody responses to group B organisms have been detected by passive hemagglutination (1, 7), latex agglutination (21), immunofluorescence (1), and bactericidal assay (6, 13, 14). The bactericidal assay measures antibodies not only against capsular polysaccharides but also against other cell envelope components and preferentially measures antibodies of the IgG class (9). Both passive hemagglutination and latex agglutination methods are relatively insensitive, detecting primarily IgM antibodies. The present ELISA overcomes these disadvantages and permits measurement of immunoglobulin class-specific

TABLE 2. Inhibition of ELISA by solid-phase bound and soluble group B polysaccharide in four sera obtained from healthy adults and four sera from convalescent meningitis patients

Sera absorbed with	Mean ELISA units <sup>a</sup> (range)			
	Meningitis patients		Healthy adults	
	IgG	IgM	IgG	IgM
Unabsorbed	359 (217-416)	148 (109-218)	20 (5-42)	344 (166-579)
Alum	324 (156-461)	142 (109-204)	21 (4-49)	339 (157-580)
Soluble B polysaccharide	243 (127-296)	107 (72-170)	7 (0-26)	275 (121-501)
Alum-bound B polysaccharide	103 (66-156)	57 (30-80)	0	102 (54-217)

<sup>a</sup> ELISA units in a 1:100 serum dilution.

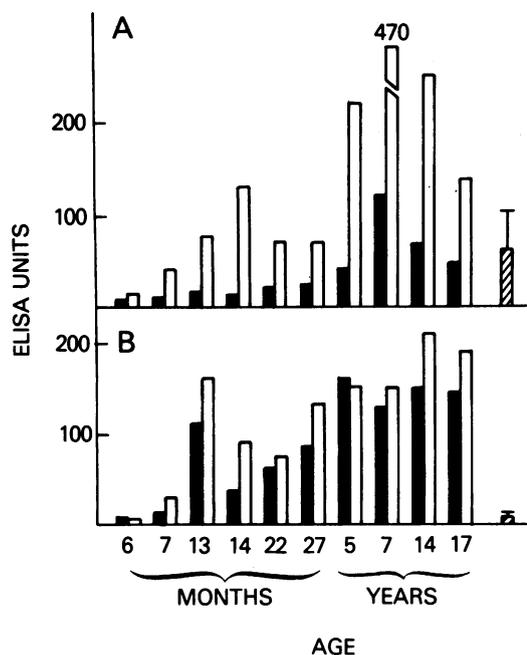


FIG. 2. IgM (A) and IgG (B) antibodies were measured by ELISA in acute and convalescent sera (diluted 1:200) obtained from 10 patients with group B meningococcal meningitis (acute sera, solid bars; convalescent sera, open bars) and in 10 sera from healthy adults (hatched bars).

antibodies directed against meningococcal polysaccharides.

The acidic meningococcal polysaccharides attached well to the basic polyamino acids arginine, lysine, and ornithine. Precoating with polylysine, which has reactive epsilon-amino groups, gave high overall antibody values with low background for the five meningococcal polysaccharides studied. Polyhistidine was not reactive, since less than 10% of histidine molecules are positively charged at pH 7.0. Polylysine has been used to fix negatively charged erythrocytes to polystyrene microtiter plates (16) and to fix a polysaccharide antigen to polystyrene for measurement of *Schistosoma mansoni* antibodies by ELISA (15). The polysaccharide and polylysine concentrations used for coating polystyrene cuvettes were about 100 times lower in our method, and no pretreatment of cuvettes with sulfuric acid was needed before coating them with polylysine. Gray (12) covalently bound pneumococcal and streptococcal polysaccharides to polylysine with cyanuric acid used as the coupling agent and showed that these complexes attach easily to polystyrene tubes and that only small amounts of polysaccharides are needed for sensitization. It is how-

ever possible that covalent binding of polysaccharides to polylysine may alter their immunological reactivity, whereas the ionic binding between polylysine and polysaccharide in our coating method may have less effect upon the antigenic configuration of polysaccharide molecules.

Artenstein et al. (1) have shown that 49, 28, and 81% of young adults have negative or very low hemagglutination titers against group A, B, and C meningococcal polysaccharides, respectively. They also showed that hemagglutinating antibodies against group B polysaccharide present in the mother are not found in the fetal serum. These results suggested that IgM is the predominant antibody class to group B polysaccharide (1). We found low levels of IgG antibodies and relatively high levels of IgM antibodies in most normal sera.

Antibody responses to group B polysaccharide in patients with group B meningococcal meningitis were also predominantly IgM. The increase in IgG antibodies between acute and convalescent sera was less than twofold, although the relative IgG antibody levels, expressed as ELISA units, were usually higher than IgM antibody levels in acute-phase serum samples. Both IgG and IgM antibodies could be removed by absorption with highly purified group B polysaccharide. The explanation for the unexpectedly high IgG antibody levels in acute sera compared with those of healthy adults may be that some antigenic determinants in the polysaccharide molecule induce a rapid IgG response during the period of contact with the organism before the onset of clinical symptoms, whereas other determinants induce a T-cell-independent IgM response after the bacteria have invaded the host. The intensity of antibody responses seems to be age dependent. Older children responded better than infants did to infection, and antibody levels in acute sera increased with the age of the patient. The age-dependent increase of responsiveness has also been shown for group A and C meningococci and *Haemophilus influenzae* type b in cases of meningitis caused by these bacteria (18).

We found that inhibition of antibody activity in ELISA was more effective when alum-bound rather than soluble group B polysaccharide preparations were used, suggesting that antibodies have higher affinity to solid-phase-bound group B polysaccharide. This finding also indicates that the bound group B polysaccharide is more antigenic than is soluble polysaccharide. Antibody responses to this polysaccharide are weak in natural infection (1, 21, 25) and absent when polysaccharide is given as a purified high-molecular-weight vaccine (22). Our results show that when presented on the surface of the bacte-

ria, the group B polysaccharide is immunogenic even in young children. Group B polysaccharide, presented as a noncovalent polysaccharide-outer membrane protein complex, was also found to be immunogenic in humans (5, 24). Thus, presentation of the group B polysaccharide to the host on the surface of bacteria during infection or bound to outer membrane components in the vaccine makes the polysaccharide immunogenic, just as binding the group B polysaccharide to a solid phase increases its antigenicity.

Differences between individual sera in the affinity of antibodies can be seen as differences in the slopes of OD-versus-serum-dilution curves (17). In our assay, these curves were parallel for both IgG and IgM antibodies against group B polysaccharide in sera obtained from healthy adults and patients. It has been shown for pneumococcal polysaccharides that there is no marked variation of the slopes between the curves of different sera when ELISA is used for measuring class-specific antibodies (17).

The use of polylysine as a precoating agent overcomes the problems of fixing negatively charged polysaccharide antigens to polystyrene. The present ELISA method may be applied for detection of antibodies against various negatively charged bacterial polysaccharides. We recently used this method to determine antibody responses to the group B polysaccharide in vaccinees, and it has proven to be a valuable tool for evaluating the immunogenicity of different group B meningococcal vaccine preparations (manuscript in preparation).

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