A Modified Enzyme-Linked Immunosorbent Assay for Measurement of Antibody Responses to Meningococcal C Polysaccharide That Correlate with Bactericidal Responses

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The standardized enzyme-linked immunosorbent assay (ELISA) for measurement of serum immunoglobulin G (IgG) antibody responses to meningococcal C polysaccharide has been modified to employ assay conditions that ensure specificity and favor detection primarily of high-avidity antibodies. The modified and standard assays were used to measure IgG antibody concentrations in sera of toddlers vaccinated with meningococcal polysaccharide vaccine or a meningococcal C conjugate vaccine. The results were compared to the respective complement-mediated bactericidal antibody titers. In sera obtained after one or two doses of vaccine, the correlation coefficients, r, for the results of the standard assay and bactericidal antibody titers were 0.45 and 0.29, compared to 0.85 and 0.87, respectively, for the modified assay. With the standard assay, there were no significant differences between the geometric mean antibody responses of the two vaccine groups. In contrast, with the modified assay, 5- to 20-fold higher postvaccination antibody concentrations were measured in the conjugate than in the polysaccharide group. Importantly, the results of the modified assay, but not the standard ELISA, paralleled the respective geometric mean bactericidal antibody titers. Thus, by employing conditions that favor detection of higher-avidity IgG antibody, the modified ELISA provides results that correlate closely with measurements of antibody functional activity that are thought to be important in protection against meningococcal disease.

Considerable efforts have been made to develop standardized protocols for quantifying serum antibody responses to meningococcal C vaccination (7, 19). The results of a multicenter study demonstrated that one can obtain reproducible measurements of antiscapsular antibody concentrations by a standardized enzyme-linked immunosorbent assay (ELISA) (7). However, in some instances, the ability to extrapolate from measurements of antiscapsular antibody concentrations in serum to predict bactericidal antibody titers appears to be limited. For example, in infants or young children who receive meningococcal polysaccharide vaccine, one can detect high serum antibody responses to group C polysaccharide by ELISA in the absence of detectable bactericidal antibody titers (5, 14, 20). The most likely explanation is that administration of the plain polysaccharide vaccine to infants and young children elicits principally low-avidity antibodies. These antibodies are detected by ELISA but appear to be less active in bactericidal function assays than higher-avidity antiscapsular antibodies (24). It is likely that the standard meningococcal C ELISA detects both high- and low-avidity antibodies. Hence, for vaccines eliciting primarily low-avidity antibody, there can be high antibody responses measured by this assay but low or absent bactericidal antibody.

Although there are substantial clinical and epidemiologic data indicating that serum bactericidal activity is a good surrogate for protection against meningococcal disease (6, 8), there are also a number of problems in developing reliable and meaningful bactericidal activity assays. These include a large degree of variability in results, depending upon the choice of bacterial strain, bacterial growth conditions, buffer, or complement source (18, 19, 27). In contrast, antibody-binding assays such as ELISAs are easier to standardize. However, to have clinically meaningful results, it is important to choose assay conditions that permit assessment of functionally active antibodies that are likely to confer protection. Toward this goal, we have developed a modified immunoglobulin G (IgG) ELISA to measure concentrations of antiscapsular antibody to meningococcal C polysaccharide in serum. As described below, we chose assay conditions that favor detection primarily of high-avidity antibodies (17). Our hypothesis was that by measuring such antibodies selectively, the results of the modified assay would correlate more closely with the results of the bactericidal activity assay than those obtained with the conventional ELISA. The purposes of this report are to describe this modified ELISA procedure and to present the results of studies using this assay to measure IgG antiscapsular antibody concentrations in stored serum samples from toddlers immunized with either meningococcal polysaccharide vaccine or an investigational meningococcal C conjugate vaccine. The same sera also were assayed by using the standardized IgG ELISA (7). The results of each of these antibody binding assays were compared to the respective bactericidal antibody titers.

MATERIALS AND METHODS

Standard ELISA. A detailed description of the standard ELISA procedure for measurement of concentrations of IgG antibody to meningococcal C polysaccharide in serum has been previously given (7). This assay uses a mixture of methylated human serum albumin and meningococcal C polysaccharide as the solid-phase antigen. Alkaline phosphatase-conjugated anti-human IgG mouse monoclonal antibody (clone HP6043) is used to detect bound IgG antibody. The limit of antibody detection in this assay is 0.02 μg/ml.

Modified ELISA procedure. (i) Derivatized polysaccharide. Meningococcal C polysaccharide (lot 84) was provided by Chiron Vaccines S.p.A. (Siena, Italy). The polysaccharide was derivatized with adipic acid dihydrazide (ADH) by the...
carbodiimide method (4). Briefly, 5-μg/ml polysaccharide was reacted with 0.5 M ADH (Sigma, St. Louis, Mo.) in the presence of 0.1 M 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide–HCl (Pierce, Rockford, Ill.) for 15 min at room temperature while the pH was maintained between 6.5 and 7.0. The derivatized polysaccharide was then washed at −20°C. Acid ninhydrin 2 reagent (Sigma) was used to determine the concentration of the polysaccharide in the final product (26), and the extent of ADH incorporation was measured by using the trinitrobenzene sulfonic acid (Sigma) assay (25). The typical antigen lots used had a polysaccharide-to-ADH ratio of 25 μg of ADH per μg of meningococcal C polysaccharide.

(ii) Assay method. Microtiter plate wells (Immulon II; Dynatech, Chantilly, Va.) were coated with 100 μl of 1-μg/ml test antigen and incubated for 1 h at 37°C in a humidified box. The solution was aspirated, and the wells were washed three times with PBS (SkanWasher 100; Skantron Instruments, Inc., Sterling, Va.) and blocked for 1 h at room temperature with 1% bovine serum albumin (BSA, Sigma) in blocking buffer (PBS, 0.02% sodium azide, pH 7.4 [Sigma]). The plates were washed three times with wash buffer (PBS, 0.1% Tween 20 [Sigma], 0.02% sodium azide, pH 7.4). Test and reference sera were prediluted with serum diluting buffer (PBS, 1% BSA, 0.1% Tween 20, 75 mM ammonium thiocyanate [SCN, Sigma], 0.02% sodium azide, pH 7.4). To make the first row of wells, 200 μl of prediluted test sera or samples of an in-house reference serum pool (A289) or quality control (QC) sera (described below). The remaining wells contained 100 μl of serum diluting buffer. Serial twofold dilutions of the serum samples were prepared in the microtiter plate, resulting in a 100-μl final volume. After the determination of the optimal coating concentration, the plates were performed with four replicate plates. Serum samples in two of the replicate plates were incubated with serum diluting buffer containing soluble meningococcal C polysaccharide (final concentration, 25 μg/ml) as an inhibitor of antibody binding. Serum samples on the other two plates were incubated with serum diluting buffer alone. The microtiter plates were maintained overnight (16 to 18 h) at 4°C in a humidified box. On the following day, wells were aspirated and washed five times with PBS. To each well was added 100 μl of alkaline phosphatase-conjugated murine monoclonal anti-human IgG (clone HP6043, conjugated by American Qualex, La Mirada, Calif.). After 1 h at 37°C, the plates were washed with PBS and 100 μl of 1-mg/ml substrate (Substrate 104; Sigma) prepared in diethanolamine solution (Pierce) was added. After 30 min, the colorimetric endpoint was stopped by the addition of 25 μl of 3 N NaOH. Absorbance at 405 nm was measured with a 630-nm background filter and a 0.49 at the lowest dilution tested (1:50) were still considered positive, since an antibody concentration, multiple data points obtained from dilutions of test sera were assigned by comparison to the reference curve. To calculate the antibody concentration, the coefficient of variation of the modified ELISA performed by different operators on different days was <10%.

Bactericidal antibody procedure. The method used for measurement of bactericidal antibody titers has been previously described (19). In the present study, the complement source was normal saline (100 μl of 30% in physiological saline) containing 2.5% horse serum (HSA, RPMI). After 60 min of incubation (37°C), the plates were assayed by an ELISA (without SCN in the serum diluting buffer). This value was obtained by comparison to the titration curve generated with the Centers for Disease Control and Prevention reference serum (CDC1992, assigned a concentration of 24.1 μg/ml monoclonal anti- meningococcal C polysaccharide antibody) (11). When the in-house reference pool, A289, was reassayed in replicate in an IgG ELISA using the ELISA protocol described above, 100 μl of each antigen preparation was added to each well, and the absorbance was read at 405 nm (ELISA plate reader, Biotek Instruments, Inc., Winooski, Vt.). The absorbances were analyzed statistically as described below.

Statistical analysis. All statistical tests were conducted at the two-sided 5% significance level. For the purpose of analysis, the IgG anti-meningococcal C and bactericidal antibody concentrations in samples collected preimmunization, 2 months post-dose 1, and 1 month post-dose 2 were logarithmically transformed (base 10). Titers below the limit of detection were set to half the limit of detection. Geometric mean concentrations were calculated using standard deviation (SD) approach or variance. The coefficient of variation of the modified ELISA was defined as the ratio of the standard deviation to the mean of the antibody concentration (20). To assess the relationships between IgG ELISA (standard or modified) and bactericidal titers, scatter plots were drawn on a log scale (base 10) for each time point. Pearson correlation coefficients were computed and regression lines were determined for linear and non-linear relationships.

RESULTS

Rationale for ELISA modifications (i) Microtiter plate coating antigen. In pilot studies, meningococcal C polysaccharide mixed with methylated human albumin was adsorbed to microtiter wells as described for the standard ELISA (7). With this antigen preparation, high absorbance values were observed when serum samples from some unvaccinated children or adults were assayed. Attempts to determine the specificity of this antibody for polysaccharide binding were inconclusive, since the standard ELISA performed on meningococcal C polysaccharide was added to the serum diluting buffer, <50% inhibition of antibody binding to the solid-phase antigen was observed with some serum samples (data not shown). Therefore, to ensure antibody binding specificity, alternative means for measuring meningococcal polysaccharide to achieve the solid phase were explored. In the process of derivatizing the polysaccharide with biotin by using adipic acid, we observed that the derivatized polysaccharide, in the absence of biotin, bound to untreated polystyrene wells at low optimal coating concentrations (100 μl of a 1.0-μg/ml solution). As shown in Fig. 1, the specificity of meningococcal C antibody binding to this solid-phase antigen appeared to be very high, as determined by dose-response inhibition of antibody binding of a variety of pre- and postvaccination serum pools in the presence of soluble meningococcal C polysaccharide. Therefore, for the modified ELISA, we chose to use the adipic acid-derivatized polysaccharide as the solid-phase antigen.

(ii) Blocking buffer. Several blocking buffers were evaluated to determine which formulation would yield the lowest background titers when nonimmune sera were assayed in different microtiter plates. Among those tried, 1% BSA (radioimmunoassay reagent grade) in the absence of a nonionic detergent, such as Brij, was equivalent or superior to other blocking buffers.
buffers, as determined by the lowest background OD values (data not shown).

(iii) Use of SCN in the serum diluting buffer. Antibody binding in the presence of increasing SCN concentrations has been reported to correlate directly with antibody avidity (17). Therefore, the chaotropic agent ammonium SCN, which inhibits antigen-antibody interactions in a dose-dependent fashion, was included in the serum dilution buffer in the modified ELISA in an attempt to select for high-avidity antibody binding. Three immune serum pools were prediluted to an antibody concentration that yielded an OD of approximately 1.0 when assayed in the absence of ammonium SCN. Figure 2 shows the results of a representative experiment in which we assessed the effect of increasing concentrations of SCN in the serum diluting buffer on the binding of IgG anti-meningococcal C antibody to the solid-phase antigen. The three pools selected were as follows. Serum pool A289, described further below, is the in-house reference pool prepared from sera of vaccinated adults. The two toddler serum pools were prepared from postvaccination sera from individuals who showed IgG anticapsular antibody responses to vaccination with meningococcal polysaccharide vaccine or meningococcal C conjugate vaccine (concentrations of <0.4 U/ml in serum obtained before vaccination increasing to ≥1.8 U/ml in postvaccination sera, as measured in the absence of SCN in the diluting buffer). Toddler pool 1 (9 subjects) contained postvaccination sera that were selected based on the presence of bactericidal antibody titers of ≥1:8, whereas toddler pool 2 (10 subjects) contained postvaccination sera that were selected based on undetectable bactericidal activity (titers of <1:8). We assumed that toddler pool 1 contained higher-avidity IgG anticapsular antibodies, while toddler pool 2 contained primarily IgG antibodies with lower avidity. A concentration of SCN was sought that would minimally decrease the IgG ELISA binding of toddler pool 1 while significantly decreasing the ELISA titers in toddler pool 2. As shown in Fig. 2, the desired results occurred at a concentration of approximately 75 mM SCN. This concentration of SCN was selected for use in the serum diluting buffer in the modified IgG ELISA.

Relationship of IgG antibody concentration to bactericidal antibody titer: comparison of the two ELISA methods. (i) Preimmune sera (naturally induced antibody). Figure 3 (top panel) shows the relationship between IgG anti-meningococcal C antibody concentrations measured in preimmune sera by the standard ELISA and the respective bactericidal antibody titers measured by using human complement. The IgG antibody concentrations measured with the standard ELISA ranged from 0.02 to 24 μg/ml. Of the 68 samples tested, 7 (10%) had detectable bactericidal antibody titers of 1:8, and none had titers of >1:8. In this collection of preimmune serum samples, there was no discernible relationship between the magnitude of the IgG anticapsular antibody concentrations and the re-
spective bactericidal antibody titers. When these sera were assayed by the modified ELISA, none had detectable IgG antcapsular antibody (Fig. 3, bottom panel), including the seven samples with bactericidal activity. Note that the lower limit of antibody detection in the modified ELISA is 0.4 U/ml, compared to 0.02 mg/ml with the standard ELISA.

(ii) Serum samples obtained 2 months post-dose 1. Figure 4 (top panel) shows the relationship between the respective IgG antcapsular antibody concentrations measured with the standard ELISA and the bactericidal antibody titers measured in post-dose 1 toddler sera. The data are stratified by vaccine group (conjugate, plain polysaccharide). For both vaccine groups, there are highly significant ($P < 0.001$) coefficients of correlation between the respective ELISA and bactericidal results (polysaccharide vaccine, $r = 0.73$; conjugate vaccine, $r = 0.53$). However, when the data from the two vaccine groups are combined, the overall correlation coefficient is lower ($r = 0.45$, $P < 0.001$). The reason for the lower overall correlation is that the $y$ intercepts of the respective regression lines for the two vaccine groups are separated by nearly a log. The most likely explanation is that affinity maturation of the antibody is elicited in response to dose 2 of the conjugate but not in response to the second dose of the plain polysaccharide vaccine. The resulting overall correlation coefficient, $r$, for the combined data after dose 2 is 0.29 ($P < 0.05$).

Figure 4 (bottom panel) shows the relationship between IgG antibody concentrations in post-dose 2 sera measured by the modified IgG ELISA and the corresponding bactericidal antibody titers. The correlation coefficient, $r$, for both the polysaccharide and conjugate groups is 0.72. For the combined data, the correlation coefficient is 0.87 ($P < 0.001$ for all three $r$ values). Thus, for both post-dose 1 and post-dose 2 sera, the

Serum samples obtained 1 month post-dose 2. Figure 5 (top panel) shows the relationship between IgG antcapsular antibody concentrations measured with the standard ELISA and the bactericidal antibody titers measured in post-dose 2 toddler sera. The correlation coefficient for the polysaccharide group is 0.58, and that for the conjugate group is 0.59 ($P < 0.001$ for both). However, the $y$ intercepts of the regression lines for the two vaccine groups are even further apart (i.e., nearly 2 logs) than the corresponding data obtained post-dose 1. The most likely explanation is that affinity maturation of the antibody is elicited in response to dose 2 of the conjugate but not in response to the second dose of the plain polysaccharide vaccine. The resulting overall correlation coefficient, $r$, for the combined data after dose 2 is 0.29 ($P < 0.05$).

Figure 5 (bottom panel) shows the relationship between IgG antibody concentrations in post-dose 2 sera measured by the modified IgG ELISA and the corresponding bactericidal antibody titers. The correlation coefficient, $r$, for both the polysaccharide and conjugate groups is 0.72. For the combined data, the correlation coefficient is 0.87 ($P < 0.001$ for all three $r$ values). Thus, for both post-dose 1 and post-dose 2 sera, the

![FIG. 3. Relationship between concentrations of IgG antibody to meningococcal C polysaccharide and bactericidal activity titers measured in prevaccination sera of toddlers 15 to 23 months of age. Except where noted, each point represents the value of a single sample.](http://cvl.asm.org/)

![FIG. 4. Relationship between concentrations of IgG antibody to meningococcal C polysaccharide and bactericidal activity titers measured in serum samples obtained 2 months after vaccination of toddlers with meningococcal polysaccharide vaccine or meningococcal C conjugate vaccine.](http://cvl.asm.org/)
modified ELISA results provide a better prediction of bactericidal antibody titers than do the standard ELISA results.

**Geometric mean antibody concentrations as assessed by the different assays.** Table 1 summarizes the geometric mean IgG antibody concentrations and bactericidal antibody titers as measured by the different assays. The discrepant results of the different IgG ELISAs are striking. For example, with the standard ELISA, there is no significant difference in the respective geometric mean IgG antibody concentrations in serum between the conjugate and polysaccharide vaccine groups after dose 1 (5.1 versus 7.0 μg/ml) or after dose 2 (9.3 versus 9.7 μg/ml). In contrast, with the modified ELISA, we observed a nearly fivefold higher IgG response in the conjugate group after dose 1 (4.8 versus 1.0 U/ml, \( P < 0.001 \)) and a 20-fold higher antibody concentration after dose 2 (21 versus 1.2 U/ml, \( P < 0.001 \)). As shown in Table 1, the respective geometric mean bactericidal antibody titers paralleled the modified IgG ELISA results and not the standard IgG ELISA results.

**DISCUSSION**

The data of Goldschneider et al. (8) from classic studies performed in the 1960s with military recruits demonstrated the importance of complement-mediated serum bacteriolysis in predicting protection against meningococcal C disease. These and other data (summarized by Frasch in 1995 [6]) provide the basis for using the bactericidal activity assay as a means of assessing protective immunity to meningococcal disease. However, the bactericidal activity assay is very labor-intensive, and even minor variations in the procedure can greatly affect the reproducibility of results (8, 18, 19, 27). For this reason, antibody-binding assays, such as the ELISA, offer a more convenient and reproducible method for quantifying immune responses to vaccination. The question, however, is whether data obtained from an antibody-binding assay can be used as a surrogate to predict bactericidal antibody responses.

The results of previous studies correlating the magnitude of the antcapsular antibody responses to meningococcal C polysaccharide measured by the standard ELISA with bactericidal antibody responses provide conflicting data. In general, higher correlations have been observed when assaying sera from immunized adults (3) or from younger individuals given a single vaccine (12). In contrast, lower correlations have been observed when assaying sera of vaccinated infants or toddlers (13), particularly when the sera are from studies comparing a polysaccharide to a conjugate vaccine (5, 14). The most likely explanation for the high correlations observed in some studies and the low correlations observed in others is the existence of differences in avidity between the antibodies measured in the particular studies. For example, when the analysis is limited to sera from vaccinated adults, the avidity of the antibody populations is likely to be higher and more homogeneous than when sera from infants and children given more than one type of vaccine are assayed. The present analysis is consistent with this explanation. Specifically, with the standard ELISA, higher correlation coefficients were observed when the analysis was limited to one of the two vaccine groups than when coefficients of correlation with bactericidal antibody titers were measured by using combined data from the two vaccine groups. In contrast, higher respective coefficients of correlation with bactericidal antibody titers were observed with the modified ELISA than with the standard ELISA, particularly when the data from the two vaccine groups were combined and analyzed together.

The most likely explanation for the improved performance of the modified ELISA is the use of SCN in the serum diluting

<p>| Table 1. Antibody concentrations in sera of toddlers vaccinated with meningococcal C conjugate vaccine or meningococcal polysaccharide vaccine, as assessed by different assays. |</p>
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<th>Assay, method, and vaccine</th>
<th>Geometric mean</th>
<th>Probability&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Conjugate vaccine</td>
<td>4.4</td>
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<sup>a</sup> Serum samples were selected from toddlers showing a wide range of IgG antibody responses to vaccination. There were 35 subjects in the polysaccharide group and 35 in the conjugate group (see Materials and Methods).

<sup>b</sup> Comparing respective geometric mean antibody concentrations or titers elicited by the two vaccines.

<sup>c</sup> In micrograms per milliliter.

<sup>d</sup> In units per milliliter.

<sup>e</sup> Reciprocal of titer.
buffer, which favors detection of high-avidity over low-avidity antibodies (17). In previous studies of Haemophilus influenzae type b anticapsular antibodies, higher-avidity antibodies were found to be more active than low-avidity antibodies in eliciting complement-mediated bactericidal activity (1, 24), in opsonization (1), or in conferring protection against experimental type b Haemophilus bacteremia (10, 15). Note, however, that two other changes were incorporated into the modified ELISA that could have contributed to the improved performance of the assay, i.e., the use of a novel solid-phase derivatized polysaccharide antigen and the use of replicate microtiter plates in assay, i.e., the use of a novel solid-phase derivatized polysaccharide antigen and the use of replicate microtiter plates in which specificity of antibody binding is assessed by subtracting the respective absorbance values obtained with each serum sample diluted with buffer containing soluble meningococcal polysaccharide inhibitor from the corresponding value obtained from the sample diluted with buffer alone. Of these two modifications, the least important is the use of the soluble meningococcal C polysaccharide inhibitor as a control for IgG antibody binding specificity, since inhibition by the soluble polysaccharide with the assay conditions used is >90% for nearly all of the samples tested (data not shown). (Note, however, that this inhibition control is important when using the modified ELISA to measure IgM or total Ig anticapsular antibody concentrations, where nonspecific binding is more of a problem [9 unpublished data].)

The present study did not address the potential importance of low-avidity anticapsular antibodies in conferring protection against meningococcal disease. Although not apparently as active as high-avidity antibodies in the bactericidal activity assay, production of low-avidity antibodies could confer protection by being present in sufficiently high concentrations to activate complement-mediated bacteriolysis or opsonization. Additional experimental and epidemiological studies are needed to define better the role of low-avidity antibodies in host protection.

Finally, it is important to note that other factors, in addition to antibody avidity, can affect the ability of antibodies to activate complement-mediated bacteriolysis. These include IgM responses (18, 23). IgG subclass distribution (2), and antibody variable region gene expression (15). Given these potential variables, it is perhaps surprising that the simple addition of a chaotropic ion to the dilution buffer to favor detection of high-avidity antibody responses had such a significant influence in improving the correlation between the respective IgG ELISA results and the bactericidal antibody titers.

In summary, the close correspondence between the magnitude of the anticapsular antibody response measured by the modified ELISA and assessment of antibody functional activity, thought to be important in protection against meningococcal disease, suggests that the modified ELISA should be useful in assessing the immunogenicity of new investigational vaccines being developed for prevention of meningococcal C disease. The present data also have implications for interpreting antibody concentrations where nonspecific binding is more of a problem.

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