Measurement of Immunoglobulin G and M Antibodies to Type 3 Pneumococcal Capsular Polysaccharide by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay was developed to measure immunoglobulin G (IgG) and IgM type 3 antipneumococcal capsular polysaccharide antibodies. The use of Fab2 fragments of rabbit antipneumococcal IgG antibody in the antibody-antigen sandwich increased the sensitivity for measuring IgM antibodies and decreased background activity in antigen-free cuvettes. This methodology detected type 3 IgM antibody responses in six of six subjects vaccinated with polyvalent pneumococcal vaccine and detected type 3 IgG antibody responses in three subjects. Results of the enzyme-linked immunosorbent assay and radioimmunoassay procedures were concordant, and postvaccination enzyme-linked immunosorbent assay IgM titers showed a stronger correlation with total radioimmunoassay antibody than did postvaccination ELISA IgG titers.

Streptococcus pneumoniae is a common cause of pneumonia, otitis media, bacteremia, and meningitis, and certain individuals, such as the splenectomized patient, have an increased risk of fatal outcome of pneumococcal disease (17). Immunization with polyvalent pneumococcal polysaccharide vaccines has been reintroduced as a method for protecting high-risk persons from pneumococcal disease (2). Present methods for evaluating the serological response to these vaccines include measurement of antibody in serum by radioimmunoassay (RIA) (16) or by enzyme-linked immunosorbent assay (ELISA) (3) and measurement of serum opsonic activity (10). Patients who benefit from pneumococcal vaccination with resulting enhancement of their defenses against pneumococcus would be expected to show increased serum levels of antibody and increased opsonic activity. We found, however, that splenectomized as well as healthy nonsplenectomized children often showed an increase in serum antibody measured by RIA without showing enhanced opsonic activity after pneumococcal vaccination (10).

To obtain additional information on the serological response to pneumococcal vaccines, we developed an ELISA technique for measuring type-specific immunoglobulin G (IgG) and IgM antibodies against type 3 pneumococcal capsular polysaccharide. Although other ELISA procedures for measuring pneumococcal polysaccharide antibody have been described (3, 5, 12, 13), we found that a modification of the method of Barrett et al. (3), which employs an antibody-antigen sandwich technique to insure an even, tightly bound antigen phase, gave the greatest sensitivity and specificity for measuring antcapsular polysaccharide antibody.

MATERIALS AND METHODS

Serum. Sixteen healthy adults (eight males and eight females) ranging in age from 21 to 39 years received a single 0.5-ml subcutaneous injection of 14-valent pneumococcal polysaccharide vaccine (Lederle lot no. 7-1367-150) containing 50 μg each of capsular antigen types 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F, and 25 (Danish nomenclature). Serum samples were taken immediately before and 1 month after vaccination. Sera were also obtained from six healthy, unvaccinated adults (four males and two females ranging in age from 22 to 55 years) on two occasions with an intervening 1-month interval. All sera were frozen immediately and stored at −70°C until assayed.

ELISA reagents. Type 3 rabbit antipneumococcal immune serum (rabbit anti-S3) was obtained from the Laboratory of the State Department of Health, Albany, N.Y. The IgG fraction was obtained by running the serum over a diethylaminoethyl column (DEAE AFFIGEL BLUE; Bio-Rad Laboratories, Richmond, Calif.). The IgG fraction was dialyzed against 0.1 M Walpole buffer (44.9 parts 0.1 N sodium acetate plus 55.1 parts 0.1 N acetic acid [pH 4.6]) and digested with 1 mg of pepsin per 100 mg of IgG. A Sephacryl G-200 column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was used to separate the Fab2 fragments from the Fc fragments. The Fab2 fraction was concentrated and stored in phosphate-buffered saline (pH 7.4) containing 0.02% NaN3 at 70°C. Rabbit anti-S3 Fab2 was
diluted 1:1,000 in phosphate-buffered saline for most experiments as the antibody portion of the sandwich.

Purified pneumococcal capsular polysaccharides, kindly supplied by Lederle Laboratories, Pearl River, N.Y., were reconstituted to 1 mg/ml in phosphate-buffered saline containing 0.02% NaN₃ and stored at 4°C. A 10-μg/ml final concentration of antigen was used to coat the cuvettes.

The IgG fractions of goat antihuman Fc fragment-specific IgG and μ chain-specific IgM (Cappel Laboratories, Cochranville, Pa.) were further purified with a DEAE AFFI-GEL BLUE column. These fractions were conjugated with horseradish peroxidase (type VE RZ30; Sigma Chemical Co., St. Louis, Mo.) by a method of the Mathiesen et al. (14). A 20-mg amount of horseradish peroxidase was dissolved in 2 ml of 0.3 M NaHCO₃ and 50 μl of 0.32% p-formaldehyde was added and stirred for 30 min at room temperature. A 2-ml volume of 0.4 M NaO₄ was added and stirred continuously for 30 min at room temperature; then 1 ml of 1 M ethylene glycol was added and the mixture was incubated with stirring for 1 h at room temperature. The solution was dialyzed against 0.01 M NaHCO₃ (pH 9.5) overnight at 4°C. A portion (15 mg) of each IgG fraction, dissolved in 1 ml of 0.01 M NaHCO₃ (pH 9.5), was added to the horseradish peroxidase dialysate with stirring for 2 h at room temperature. The solution was precooled to 4°C, and 10 mg of NaN₃ was added. The solution was left at 4°C for an additional 2 h and then dialyzed overnight at 4°C against phosphate-buffered saline. Column fractionation was performed with Sephacryl G-200. Working solutions of horseradish peroxidase-labeled goat antihuman IgG (IgG conjugate) and horseradish peroxidase-labeled goat antihuman IgM (IgM conjugate) were prepared by dialysis in phosphate-buffered saline containing 0.03% Tween 20 (Sigma Chemical Co.) and 1% bovine albumin.

The substrate employed was 0.03% H₂O₂ in citrate phosphate buffer (pH 5.0) containing 2 mg of o-phenylenediamine dihydrochloride per ml. The substrate (200 ml) was prepared immediately before addition to cuvettes.

ELISA procedure. Polystyrene microcuvettes (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) were coated with antigen by one of three techniques described below. After antigen incubation, the cuvettes were washed three times, and dilutions of serum were added and incubated at 37°C for 2 h. The cuvettes were washed three times, 0.25 ml of the IgG or IgM conjugate was added, and the mixture was incubated for 1 h at 37°C. The cuvettes were washed four times, and 0.25 ml of substrate was then added. An absorbance reading was taken at 450 nm with a Gilford EIA 50 automated spectrophotometer after 5 min of incubation at 30°C. The substrate incubation was controlled at 30°C in a temperature-controlled plexiglass box that housed the Gilford EIA 50.

RESULTS

Antigen-coated cuvettes. Purified pneumococcal polysaccharide types 1, 3, 6A, 7F, 14, 18C, 19F, and 23F were diluted in 0.1 M Na₂CO₃ containing 0.02% NaN₃ (pH 9.8) to a concentration of 10 μg/ml and were incubated in polystyrene microcuvettes (Gilford). Maximum binding and stabilization of the antigen phase occurred after overnight incubation at 37°C followed by storage for at least 10 days at 4°C. The absorbance values for antigen-free cuvettes which contained only 0.1 M Na₂CO₃ with 0.02% NaN₃ (i.e., the background absorbance) were subtracted from the absorbance values for antigen-coated cuvettes. With the IgG conjugate, background absorbance was primarily attributable to binding of the conjugate and not to binding of serum components (Table 1). With the IgM conjugate, background activity increased substantially with the addition of serum (Table 2).

Cuvettes coated with types 1, 3, 6A, 7F, 14, 18C, 19F, and 23F polysaccharides were used to measure IgG and IgM antibody activity in the pre- and postvaccination sera from 10 vaccinated and 6 unvaccinated control subjects. None of the unvaccinated control subjects showed greater than a 1.3-fold increase in IgG or IgM antibody activity to any of the antigen types. There was a marked discordance between the ELISA and RIA results on comparison of the numbers of vaccinated subjects showing significant increases in antibody activity to types 3, 6A, 14, 19F, and 23F (Table 3). Concordance was observed for types 1, 7F, and 18C. However, there was no correlation between total RIA activity

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**TABLE 1. Measurement of type 3 IgG antibody activity by three different ELISA procedures**

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Mean absorbance units ± SE for antibody activity in serum obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevaccination</td>
</tr>
<tr>
<td>C</td>
<td>0.235 ± 0.014</td>
</tr>
<tr>
<td>Ag + S + C</td>
<td>2.720 ± 0.020</td>
</tr>
<tr>
<td>S + C</td>
<td>0.174 ± 0.017</td>
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<tr>
<td>Net absorbance</td>
<td>2.546</td>
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<tr>
<td>Anti-S₁ IgG + Ag + S + C</td>
<td>2.668 ± 0.022</td>
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<tr>
<td>Net absorbance</td>
<td>0.406</td>
</tr>
<tr>
<td>Anti-S₁ IgM + S + C</td>
<td>2.262 ± 0.010</td>
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<tr>
<td>Net absorbance</td>
<td>0.508 ± 0.025</td>
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<tr>
<td>Anti-S₁ Fab₂ + Ag + S + C</td>
<td>1.411 ± 0.075</td>
</tr>
<tr>
<td>Net absorbance</td>
<td>0.903</td>
</tr>
</tbody>
</table>

* A fixed serum dilution (1:100) was used in all assays for both pre- and postvaccination samples. The antigen concentration was 10 μg/ml, the anti-S₁ IgG concentration was 10⁻⁴, and the anti-S₁ Fab₂ concentration was 10⁻³. C, IgG conjugate; Ag, type 3 capsular antigen; S, serum. Net absorbance is absorbance in antigen-coated cuvettes minus background absorbance in antigen-free cuvettes.
TABLE 2. Measurement of type 3 IgM antibody activity by three different ELISA procedures

<table>
<thead>
<tr>
<th>Reagents*</th>
<th>Mean absorbance units ± SE for antibody activity in serum obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre vaccination</td>
</tr>
<tr>
<td>C</td>
<td>0.089 ± 0.009</td>
</tr>
<tr>
<td>Ag + S + C</td>
<td>1.458 ± 0.080</td>
</tr>
<tr>
<td>S + C</td>
<td>0.230 ± 0.013</td>
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<tr>
<td>Anti-S3 IgG + Ag + S + C</td>
<td>1.228</td>
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<td></td>
<td>0.425</td>
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<tr>
<td>Anti-S3 Fab2 + Ag + S + C</td>
<td>0.732 ± 0.033</td>
</tr>
<tr>
<td>Anti-S3 Fab2 + S + C</td>
<td>0.640 ± 0.029</td>
</tr>
<tr>
<td></td>
<td>0.092</td>
</tr>
</tbody>
</table>

* A fixed serum dilution (1:100) was used in all assays for both pre- and postvaccination samples. The antigen concentration was 10 μg/ml, the anti-S3 IgM concentration was 10^-4, and the anti-S3 Fab2 concentration was 10^-3. C, IgM conjugate; Ag, type 3 capsular antigen; S, serum. Net absorbance is absorbance in antigen-coated cuvettes minus background absorbance in antigen-free cuvettes.

and the sum of IgG and IgM ELISA activity for any of the antigen types, even with types 1, 7F, and 18C. Correlation coefficients for RIA versus ELISA antibody activity for types 1, 7F, and 18C were 0.23, 0.33, and 0.25, respectively. These results indicated that pneumococcal capsular polysaccharide antigen bound to microcuvettes, but that cuvettes coated with antigen alone were not suitable for measuring a change in antcapsular antibody after vaccination.

Antibody-antigen coated cuvettes. Antigen binding was improved by using a modification of the method described by Barrett et al. (3), whereby type-specific rabbit antibody is first adsorbed to the cuvettes, and antigen is reacted with the antibody to form a stable, antigen-coated surface. To improve the sensitivity of this procedure, the IgG fraction of rabbit antiserum was isolated from a DEAE AFFI-GEL BLUE column and used to coat the cuvettes. Despite this modification, background activity in antigen-free control cuvettes (coated only with rabbit antibody) remained high with the IgG conjugate (Table 1), and very little specific antibody was measured in immune serum with the IgM conjugate (Table 2). To reduce background activity, the IgG fraction of rabbit anti-S3 was pepsin digested, and the Fab2 fragments were isolated and used to form the base of the antibody-antigen sandwich. Background activity was greatly reduced when Fab2 fragments instead of whole IgG were used in the sandwich to measure IgG antibody activity (Table 1). There was no difference in background activity when Fab2 fragments were substituted for whole IgG to measure IgM antibody activity (Table 2).

Various concentrations of rabbit anti-S3 Fab2 fragments and various concentrations of purified S3 antigen were added to cuvettes in checkerboard titrations to determine the appropriate concentrations of these reagents. A 10^-3 dilution of rabbit anti-S3 Fab2 fragments and a 10-μg/ml concentration of S3 antigen were optimum. The Fab2 fragments were incubated in cuvettes overnight at 37°C and were refrigerated at 4°C for up to 10 days with no decrease in sensitivity or specificity for measuring IgG or IgM anti-S3 antibody. The cuvettes were washed three times with 0.9% NaCl containing 0.5% Tween 20 before addition of antigen. Incubation of S3 antigen for 2 h at 37°C in the Fab2-coated cuvettes was sufficient for optimum formation of a stable antigen phase.

Absorbance readings were taken at 1-min intervals to determine the optimum time for substrate incubation. The increase in absorbance with length of substrate incubation was linear during the first 5 min of substrate incubation up to a value of 2.5. The optimum concentrations of o-phenylenediamine dihydrochloride and H2O2 were 4 mg/ml and 0.06%, respectively.

Separate experiments were performed to determine the optimum time, temperature, and concentrations of reagents to measure IgG and IgM antibodies to type 3 pneumococcal capsular
polysaccharide. The binding kinetics for both antibody classes were the same. Human serum antibody bound optimally after 2 h of incubation at 37°C in antibody-antigen-coated cuvettes. Maximum binding of the IgM and IgG conjugates occurred after 1 h of incubation at 37°C. Antigen-free cuvettes were run for each serum dilution tested to measure background activity. The concentration of human serum had no effect on the background activity measured in antigen-free cuvettes when anti-S2 Fab2 was used, but the concentration of both the IgG and IgM conjugates had a significant effect, which was directly proportional to the concentration of the conjugate used.

The uptake of type-specific antibody was studied by adding various dilutions of sera to antibody-antigen-coated cuvettes (Fig. 1). The log of net absorbance at 450 nm (absorbance in antigen-coated cuvettes minus background absorbance in antigen-free cuvettes) was linearly related to the log of the reciprocal serum dilution, and the lines obtained with various sera both before and after vaccination were approximately parallel. ELISA titers were arbitrarily defined as the reciprocal of that serum dilution which would result in an absorbance of 0.3 U. Serum dilutions which yielded absorbances of greater and less than 0.3 U were used; thus, the resulting line always intersected the reference value of 0.3 U. A serum assayed at multiple dilutions and subjected to linear regression analysis was used to establish the slope of a line relating change in absorbance units to change in reciprocal serum dilution for a particular set of determinations. Individual values were eliminated if they were beyond the linear range of the peroxidase assay (absorbance greater than 2.5), widely variant from the other cuvettes in that set, or too low for accuracy (absorbance within 0.05 U of background absorbance). ELISA titers were corrected in relation to a simultaneously assayed reference serum because of a slight variation in ELISA titers in repeated assays.

ELISA antibody titers with this procedure were highly reproducible. Within 1 day, IgG and IgM titers varied among cuvette strips within 0.16 and 0.25 log10 units, respectively; the errors were 3.0 and 5.1%, respectively. Day-to-day results varied within 0.27 log10 units for the IgG titer and within 0.57 log10 units for the IgM titer of immune serum with cuvettes set up simultaneously; the errors were 5.1 and 10.5%, respectively.

ELISA titers for vaccinated and unvaccinated subjects. Serum was obtained on two occasions, with a 1-month interval, from six unvaccinated control subjects. Three subjects had detectable type 3 IgG antibody activity, and none had measurable type 3 IgM antibody activity (Fig. 2). The mean ratio of the IgG ELISA titer in the second serum sample to that in the first sample was 0.94 ± 0.18 (one standard deviation) for the six subjects. Therefore, an increase in ELISA titer between two serum samples of at least 1.3-fold (mean ± two standard deviations) was taken to represent a significant increase in antibody activity.
FIG. 3. Type 3 antipneumococcal IgG and IgM antibody activity measured by ELISA in two serum samples obtained from six healthy adults before (open bars) and 1 month after (shaded bars) pneumococcal vaccination. The height of each bar represents the mean ELISA titer of three to six separate runs; the vertical line through the mean represents the standard error of these observations. The number over each pair of bars is the ratio of the antibody titer in the second sample to the titer in the first sample. Fab₂ antibody-antigen-coated cuvettes were used.

Serum was obtained from six healthy adult subjects before and 1 month after administration of polyvalent pneumococcal vaccine. All six subjects had measurable type 3 IgG antibody activity in the prevaccination serum sample, and three showed significant increases in antibody activity after vaccination (Fig. 3). All six subjects showed significant increases in type 3 IgM antibody activity. There was no relationship between the titer of antibody activity in the prevaccination serum and the magnitude of the antibody increase after vaccination.

There was no significant difference between pre- and postvaccination sera in the slope of the absorbance versus serum dilution linear regression curve for subjects with ELISA antibody activity (Fig. 1). The mean slope for IgG activity in prevaccination sera was 0.828 ± 0.049 (standard error), and the mean slope for IgG activity in postvaccination sera from the same patients was 1.028 ± 0.047. An analysis of this difference by the Student t test for paired data showed no significant difference (t = 0.965). The mean slope for IgM activity in prevaccination sera was 0.732 ± 0.085, and the mean slope for IgM activity in postvaccination sera was 0.948 ± 0.123. An analysis of this difference by the paired t test showed no significant difference (t = 0.882).

A strong linear correlation was observed between the sum of type 3 IgG and IgM antibody activity measured by ELISA and total antibody activity measured by RIA in the six paired sera from vaccinated subjects (r = 0.694, P = 0.012) (Fig. 4). Prevaccination antibody activity measured by RIA correlated poorly (P > 0.2) with both IgG and IgM antibody activity measured by ELISA. Postvaccination antibody activity measured by RIA correlated better with IgM antibody activity (r = 0.814, P = 0.049) than with IgG antibody activity (r = 0.750, P = 0.086).

**DISCUSSION**

RIA is the methodology presently employed most widely for measuring the immunogenicity of pneumococcal capsular polysaccharide vaccines. Measurement of serum opsonic activity, while not quantitative, reflects antibody- as well as complement-mediated serum activity against pneumococcus (11). To obtain more specific information on the immune response to pneumoccus and to pneumococcal polysaccharide, we developed an ELISA technique to measure type-specific IgG and IgM antibodies to type 3 pneumococcal polysaccharide. Several problems were encountered during the development of this procedure, the most significant of which was the failure of polystyrene microcuvettes coated with antigen alone to measure antibody produced after vaccination, despite binding of
polysaccharide antigen to the cuvettes. The ELISA procedure was further refined by using a stable antibody-bound antigen phase which proved to be simple yet sensitive and efficient for the detection of both IgG and IgM class-specific antibodies to type 3 pneumococcal capsular polysaccharide.

Barrett et al. described an ELISA procedure for measuring antipneumococcal capsular polysaccharide antibody by using an antibody-bound antigen phase (3). In our experiments, the IgG fraction of rabbit anti-S3 serum gave high background activity in antigen-free cuvettes, possibly due to anti-Fc activity in many normal human sera. We modified the procedure of Barrett et al. and improved the specificity for measuring IgG and IgM antibodies by using rabbit anti-S3 Fab2 fragments in the antibody-antigen sandwich. Some background activity persisted, however, in antigen-free as well as antibody- and antigen-free cuvettes, suggesting that the conjugate alone bound to the polystyrene surface. As the concentration of Fab2 fragments was increased, more binding occurred; a $10^{-3}$ dilution of rabbit anti-S3 Fab2 fragments was sufficient and economical. An S3 antigen concentration of 10 μg/ml gave the maximum type 3 IgG and IgM antibody activity. Others have also shown that higher and lower antigen concentrations result in a reduced ability to measure antibody activity (3, 6, 8). The decline in sensitivity when higher antigen concentrations are used may be caused by elution of antigen from the antibody-antigen complex or by elution of antigen bound loosely to the polystyrene surface during the serum incubation phase, allowing free antigen to react with serum antibody and thereby reducing the amount of antibody detected. This mechanism may explain the reduced sensitivity we observed when measuring antibody activity in prevaccination sera using cuvettes coated with antigen alone.

The type-specific pneumococcal antibody ELISA titers varied slightly in repeated assays. This variability could have been due to small temperature variances during substrate incubation, variation in antigen binding to the anti-S3 Fab2 polystyrene complex, or technical inaccuracies (7, 9). The variance values were acceptable and were as low, if not lower, as with the RIA procedure (16).

Several investigators have presented evidence suggesting that ELISA, as a purely quantitative assay, might be influenced by the affinity of the primary antibodies (1, 4, 8). Butler et al. hypothesized that the binding of the large secondary antibody-enzyme complex weakens the binding of low-affinity primary antibodies to the point of their displacement during the subsequent washing procedures (4). Others have observed a negative correlation between ELISA and RIA results and have suggested that the differences observed are due to variable antibody affinity (5). We observed a poor correlation between prevaccination ELISA titers and RIA results, but a good correlation between postvaccination IgG and IgM ELISA titers and total antibody measured by RIA. Since IgM was the predominant antibody induced by vaccination, it was not surprising that the correlation between postvaccination IgM ELISA titer and RIA activity was better than the correlation between IgG ELISA titer and RIA activity.

The slope of the regression line relating net absorbance in the ELISA procedure to serum dilution may reflect antibody affinity, since less-avid type-specific antibody might be more easily eluted from antigen during the wash before addition of substrate. The slope of this line was the same for prevaccination and postvaccination sera, suggesting that antibodies measured before and after vaccination had equal affinity for the antigen phase. Kasekela and Leinonen made a similar observation (13). These observations may have clinical importance, since high-affinity antibodies may be more protective (1). Thus, the ELISA methodology described may permit measurement of antibody affinity which may also be important in the in vitro evaluation of vaccine immunogenicity.

In summary, an ELISA procedure was developed by using an Fab2 antibody-antigen sandwich which proved to be highly sensitive and specific for measuring IgG and IgM antipneumococcal capsular polysaccharide antibody.

ACKNOWLEDGMENTS

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LITERATURE CITED


