Enzyme-Linked Immunosorbent Assay for Titration of *Haemophilus influenzae* Capsular and O Antigen Antibodies

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The enzyme-linked immunosorbent assay (ELISA) was elaborated for the detection of immunoglobulin M (IgM) and IgG antibodies against capsular and O antigens of *Haemophilus influenzae*. Purified capsular polysaccharide and lipopolysaccharide were used as antigens, with optimal coating concentrations being about 50 and 100 µg/ml, respectively. The antibody content was expressed as the highest serum dilution (−log10) showing an absorbance of 0.2 above the background level. The titers of hyperimmune sera (reference sera) ranged between 5 and 7 −log10. The sensitivity of the method was about 80 ng/ml with regard to antcapsular antibodies and 3 to 5 ng/ml with regard to anti-lipopolysaccharide antibodies. For detection of antibodies against capsular polysaccharide in sera obtained after primary immunization, ELISA was about 100-fold more sensitive than the indirect hemagglutination assay, whereas in hyperimmune sera, ELISA was about 10-fold more sensitive than the indirect hemagglutination assay. The sensitivity of ELISA for detecting antcapsular antibodies after primary and booster immunizations was 50-fold higher than that of the bactericidal assay using capsulated bacteria, whereas the sensitivity of the two methods was the same when hyperimmune sera were tested. ELISA performed with lipopolysaccharide as the antigen was about 50- and 150-fold more sensitive than the complement fixation and bactericidal assays tested with noncapsulated variants after primary injection and hyperimmunization, respectively.

The antibody response to the capsular polysaccharide (CPS) of *Haemophilus influenzae* type b has been estimated by indirect hemagglutination assay (IHA) (1, 4) and, more recently, by radioimmunoassay (1, 11). Both methods have certain disadvantages. IHA is comparatively insensitive, whereas radioimmunoassay, being sensitive, requires certain precautions in handling radioactive reagents as well as specialized equipment for measurement. In addition to the aforementioned methods, the complement-dependent bactericidal assay (BC) has been used as a test mainly for measuring the amount of antibodies against the CPS antigen (1, 5, 6a), although the bactericidal effect might, to a certain extent, be due to antibodies against other antigens as well (1, 6a). For the determination of antibodies against the cell wall antigens of noncapsulated *H. influenzae*, the BC assay (5, 6a) and a complement fixation (CF) test have been available (4).

In contrast to the above-mentioned methods, which are dependent on secondary manifestations for visualization of the antigen-antibody reaction, the primary binding between antigen and antibody may be revealed by the enzyme-linked immunosorbent assay (ELISA) (7, 15). By the use of immunoglobulin class-specific conjugates, this technique also offers a simple method for measuring and differentiating between immunoglobulin M (IgM), IgG, IgA, or secretory IgA class antibodies without laborious separation of serum or secretions. The micro-ELISA variant (12) is useful when small amounts of serum and secretion are available.

The aim of the present investigation was to elaborate suitable experimental conditions for the application of ELISA to the determination of antibodies against *H. influenzae* CPS and lipopolysaccharide (LPS) antigens. The sensitivity of ELISA for the detection of *H. influenzae* antibodies after primary and booster immunizations and hyperimmunization was compared with that of other methods such as IHA, CF, and BC.

**MATERIALS AND METHODS**

**Strains.** *H. influenzae* type a, strain Smith (Ma), *H. influenzae* type b, strain RAB (Mb), and their respective noncapsulated variants (Sa and Sb) were the same as those used in previous studies (3-5, 6a).

**Antisera.** Antisera against Ma, Mb, Sa, and Sb bacteria, obtained as described previously (5, 6a), were used in the study. The immunization schedule and the bleedings are shown in Fig. 1 and 2.
The antisera against E. coli O75:K100 and the pneumococcal strains were obtained from Statens Seruminstitut, Copenhagen, Denmark; antisera against the remaining strains were obtained from the Institute for Medical Microbiology, University of Göteborg, Göteborg, Sweden.

Antigenic preparations for ELISA. Purified CPS was prepared from the Ma and Mb supernatants of shaken 10-h-old fluid cultures in antigen-free medium (2). These preparations were designated CPS Ma and CPS Mb, respectively. A slight modification of the medium was utilized; the medium materials, casein hydrolysate and yeast autolysate, were dialyzed against distilled water at 4°C for 1 week to week (dialysis tubes; pore size, 240 nm; Kebo-Grave, Spånga, Sweden), instead of being filtered through Sephadex G-25, for obtaining low molecular medium. Salts and growth factors were added before sterilization as previously described (2). During the earlier part of the study, CPS (designated CPS I) was prepared from 10-fold-concentrated culture supernatants which were filtered through Sephadex G-150 (3). Fractions that eluted with the void volume, containing the capsular antigen together with some contaminants (LPS and probably adhering medium proteins), were pooled and further purified by diethylaminoethyl-Sepharose chromatography using an NaCl gradient (0 to 2 M). The O antigen eluted before the main part of the capsular antigen as tested by immunodiffusion analyses (16). Capsular antigen-containing fractions, with no absorbance or only absorbance below 0.03 at 260 and 280 nm, were pooled and lyophilized, constituting the CPS I antigen. Its protein content was about 1% as estimated by the method of Lowry et al. (10).

During the latter part of the study, capsular antigen (CPS Mb II) was obtained by precipitation of dialyzed culture supernatant with 0.5% cetyltrimethylammonium bromide (final concentration) at 4°C for 2 weeks. The capsular material was dissolved in 0.4 M NaCl and further purified by three repeated precipitations in 3 volumes of ethanol with solution in distilled water. Centrifugation and discarding of water-insoluble material were performed between each precipitation. The material was finally purified by filtration on a Sepharose-2B column, and fractions containing the capsular antigen, as determined by immunodiffusion analysis, were collected as shown in Fig. 3. The pooled capsular material was dialyzed and lyophilized. Its protein content was estimated at 0.2% by the method of Lowry et al. The preparation did not contain detectable amounts of O antigen, as estimated by immunodiffusion analysis with 1 mg of CPS Mb II per ml. The immunodiffusion method indicated about 15 μg of LPS per ml.

LPS was prepared from two noncapsulated variants, Sa and Sb, of the capsulated strains (5, 6a) by hot phenol-water extraction (17). The crude LPS extracts were treated with ribonuclease (type 1-A; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 0.1 mg of enzyme per mg of LPS (pH 7.8) for 7 h and then centrifuged at 105,000 × g for 4 h. These preparations were designated LPS Sa and LPS Sb, respectively. One part of LPS was lyophilized to estimate the concentration of antigen. The part of LPS used for ELISA...
was not lyophilized because of incomplete solubility of *H. influenzae* LPS after lyophilization. The protein content was 2% as tested by the method of Lowry et al.

Serological methods. (i) ELISA. ELISA was performed essentially as described by Engvall and Perlmann (8).

(a) Antigen-coated tubes. Disposable polystyrene tubes (Heger Plastic AB, Stallarholmen, Sweden) were coated with 0.5 or 1 ml of antigen solutions in concentrations ranging from 1 to 1,000 μg/ml. The optimal antigen concentrations were determined for titration of IgM as well as IgG antibodies. The tubes were coated overnight at 37°C and then kept at 4°C in the sealed tubes for from a few days up to 3 months to evaluate the efficiency and stability of the coating.

(b) Enzyme-conjugated anti-immunoglobulin. Sheep or swine antisera specific to rabbit IgG and IgM immunoglobulins (Nordic Immunological Laboratories, Tilburg, The Netherlands) were conjugated with alkaline phosphatase (calf intestinal mucosa, type VII; Sigma Chemical Co.) with the aid of glutaraldehyde as described by Holmgren and Svennerholm (8). The suitable concentration of the conjugates and the enzyme-substrate reaction time were determined with hyperimmune reference sera. The conjugates could be used generally in dilutions of about 1:300 for anti-IgM and 1:400 for anti-IgG, with the enzyme-substrate reaction time being about 60 min.

(c) Standardization of ELISA. Rabbit hyperimmune serum sera were used as references in all experiments for the different serological systems. The enzyme-substrate reactions were allowed to continue until the tubes containing the reference serum dilutions had reached appropriate absorbance values, giving approximately the same endpoint titers (see Table 1) at each separate experiment. An antigen-coated tube incubated with the enzyme substrate (blank) and another tube incubated with conjugates and phosphate-buffered saline instead of serum (background absorbance) were included as controls in each set of experiments.

(d) Micro-ELISA. Micro-ELISA was performed on microplates (Dynatech, Micro-ELISA; Nova Kemi AB, Enskede, Sweden) using a total reactant volume of 0.2 ml. Otherwise, experimental conditions were as described above. Absorbances were registered by an automatic spectrophotometer (Titertek Multiskan; Flow Laboratories, Solna, Sweden). The reference anti-Ma, anti-Mb, anti-Sa, and anti-Sb sera were tested simultaneously by micro- and macro-ELISA for comparison and standardization of experimental conditions so as to obtain comparable sensitivity.

(ii) Quantitative precipitin determination. Quantitative precipitin determination (9) was performed to estimate the amount of specific antibodies against CPS Ma, CPS Mb, LPS Sa, and LPS Sb in homologous hyperimmune antisera. A 0.2- or 0.25-ml amount of serum was mixed with equal volumes of antigen dilutions with concentrations ranging from 0.002 to 1 mg incubated for 1 h at 37°C and was then kept about 1 week at 4°C. The maximum amount of precipitated antibodies was determined by the method of Lowry et al.

(iii) Absorption of serum. The anti-Mb serum diluted 1:1,000 was mixed with various dilutions of CPS Mb I or CPS Mb II antigens ranging in concentrations from 0.2 to 200 μg. After incubation for 1 h at 37°C, the tubes were stored at 2°C for about 1 week. The supernatants were then tested for remaining antibodies by micro-ELISA using the IgG conjugate. The antigen concentration giving 50% reduction of the extinction at 400 nm was used as a measure of the inhibiting capacity.

(iv) Other serological methods. BC, used for determination of bactericidal antibodies against capsulated and noncapsulated bacteria, IHA, used for measuring antibodies against capsular antigen, and CF, used for measuring antibodies against cell wall antigens, were performed as previously described (4, 5).

Statistical methods. Calculation of mean and standard deviation of titer values was performed according to standard methods.

RESULTS

A series of experiments was performed to study various factors having a possible influence on the results obtained by ELISA as used for detection of antibodies against *H. influenzae* CPS and LPS.

Antigen concentration and coating time. For CPS Ma I and CPS Mb I, increasing absorbance was registered when increasing concentrations of antigen were used for the coating of the tubes, with no leveling of absorbance being reached up to an antigen concentration of 1 mg/ml (Fig. 4). To obtain the highest titers of the homologous reference sera, coating with CPS Ma I preparations should be performed at 4°C.
for 1 to 2 days, whereas coating with CPS Mb I should be performed for about 2 to 3 weeks. When the CPS I antigen was used for coating at a concentration of 100 to 200 μg/ml, the IgG and IgM titers of the reference sera were about the same as those observed when higher concentrations of antigen were used for coating. In the earlier part of the study, 100 to 200 μg of CPS I per ml was found to be suitable for determination of both IgG and IgM antibodies.

The CPS II antigen preparations adsorbed to the tubes in lower concentration than did CPS I. Optimal coating was obtained by using about 50 μg/ml as determined by IgG conjugate (Fig. 4). In addition, the CPS II preparations adsorbed more effectively (overnight incubation at 37°C and 1 to 2 days at 4°C) than did the CPS I. To save material, a suboptimal concentration of 30 μg/ml was utilized for further assays.

The inhibition study with CPS Mb I and CPS Mb II antigen preparations and an anti-Mb serum dilution of 1:1,000 revealed that the inhibitory concentrations causing 50% reduction of extinction at 400 nm were about 250 μg/ml for the CPS Mb I and about 30 μg/ml for the CPS Mb II antigen preparations.

Optimal coating of tubes with LPS was obtained by using a solution containing 100 μg/ml as tested with 10-fold dilutions of LPS antigen (Fig. 5).

**FIG. 4.** Determination of optimal amounts of CPS Ma I, CPS Mb I, CPS Ma II, and CPS Mb II antigens for coating of tubes. The anti-Ma and anti-Mb sera were diluted 1:1,000. A second anti-Mb serum was tested with CPS Mb II and was diluted 1:100. The IgG conjugate was used in the test.

**FIG. 5.** Determination of optimal amount of LPS Sb antigen for coating of tubes. The anti-Sb serum was diluted 1:1,000. The IgG conjugate was used in the test.

**FIG. 6.** ELISA titration curves for IgG and IgM class antibodies as obtained with rabbit antiserum tested against CPS Mb II antigen. (a) After primary injection. IgG titer, <1 -log_{10}; IgM titer, 2.5 -log_{10}. (b) After hyperimmunization (reference serum). IgG titer, 4.5 -log_{10}; IgM titer, 3.8 -log_{10}.

**Principles for quantitation of antibodies.**

The steepness of the linear portions of the titration curves increased during the course of the immunization. This increase was pronounced for IgG antibodies. The IgG curves were steeper than those obtained with anti-IgM conjugates. Figures 6a and 6b give representative examples of the titration curves of sera obtained after primary injection and hyperimmunization using CPS Mb II antigen. For ELISA performed with *H. influenzae* CPS and LPS antigens, the antibody content was expressed as the -log_{10} of the serum dilution (titer) which showed an absor-
bance of 0.2 above the background level. The background absorbance for the CPS as well as the LPS antigens was generally low, about 0.01 to 0.07.

Precision of ELISA. The rabbit reference serum sample was titrated 10 times on separate days with two lots of conjugates and two CPS Mb II antigen preparations. The standard deviation calculated from these experiments was 0.21 \(-\log_{10}\).

Sensitivity and specificity of ELISA. To estimate the sensitivity of ELISA, quantitation of the specific antibody content in rabbit hyperimmune sera was performed in parallel by means of the quantitative precipitation test. The lowest amount of antibodies detectable with ELISA for *H. influenzae* CPS and LPS was calculated from the maximum amount of precipitated antibodies and the corresponding ELISA titer by using IgG conjugate. The lowest amount of antibodies detectable with ELISA was about 80 ng/ml for anti-CPS Ma and Mb and about 3 to 5 ng/ml for anti-LPS Sa and Sb (Table 1).

To study the specificity of ELISA concerning anti-capsular b antibodies, in addition to the homologous antisera, a number of antisera against various gram-negative and gram-positive bacteria, including five remaining types of *H. influenzae*, were tested for presence of IgG antibodies against CPS Mb II antigen by micro-ELISA (Table 2). Demonstrable titers were obtained in sera against *H. influenzae* type a (about 2.4 \(-\log_{10}\)), *S. pneumoniae* type 6 (about 2.2 \(-\log_{10}\)), and *E. coli* O75:K100 (about 3.4 \(-\log_{10}\)).

As regards antibodies against LPS Sb antigen, the aforementioned sera were employed in the study. No demonstrable titers were noted in any of them.

<table>
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<tr>
<th>Table 1. Comparative IgG antibody class determination of anti-Ma, anti-Mb, anti-Sa, and anti-Sb hyperimmune sera tested against homologous CPS (I and II) and LPS antigens, respectively</th>
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* CPS I used in ELISA and quantitative precipitation.

Comparison of macro- and micro-ELISA. Parallel experiments were performed with reference sera by using optimal concentrations of the corresponding antigens and the same conjugate concentration and enzyme-substrate reaction time. The greatest differences recorded between results obtained by macro- and micro-ELISA techniques were limited to 0.2 \(-\log_{10}\) when CPS and LPS antisera were used in the assays. There was no tendency for any technique to give generally higher values.

Antibody response in rabbits against capsulated bacteria studied by means of ELISA, IHA, and BC assays. The four rabbits immunized with capsulated bacteria had no demonstrable antibodies against CPS Ma and CPS Mb in their preimmunum serum samples as revealed by ELISA. The primary immunization with Ma or Mb bacteria caused an IgM antibody response within 2 to 4 days, whereas an IgG antibody response was not demonstrable until 7 to 14 days. The IgM titers rapidly decreased to low levels before the booster dose, in contrast to the IgG titers, which increased continuously.

The booster dose caused an IgM antibody response of the same magnitude as that obtained after the primary immunization, whereas the IgG titer levels increased only slightly. During the hyperimmunization course, the IgM and IgG antibody levels increased gradually to titer values of about 4 and 5 \(-\log_{10}\), respectively. Figure 1 gives the development of ELISA IgM and IgG antibody titers in a rabbit given Mb bacteria, the titer development being representative for

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<th>Table 2. ELISA titers ((-\log_{10}) against CPS Mb II antigen in antisera against various bacteria</th>
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<td><em>P. vulgaris</em></td>
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the other rabbits as well.

The antisera were also tested against LPS Sb antigen. The development of IgM and IgG antibodies was similar to that described below for the antisera against noncapsulated bacteria. However, the titers were about 10- to 100-fold lower in the hyperimmune sera against capsulated bacteria than in sera against the corresponding noncapsulated variant.

In addition, the IHA and BC titers are given for comparison of sensitivity. For the four sera, the IHA titers were 50- to 100-fold lower after the primary immunization than the corresponding ELISA titers. During the hyperimmunization course, the IHA titers increased to about the same levels as ELISA IgM titers, being about 70-fold lower than the ELISA IgG titers (Fig. 1). The BC titers were 10- to 50-fold lower than the corresponding ELISA IgM titers after the primary immunization. At the end of the hyperimmunization course, very high BC titers were obtained, showing even higher levels than the ELISA IgG in the corresponding serum samples (Fig. 1).

**Antibody response in rabbits against noncapsulated bacteria studied by means of ELISA, CF, and BC assays.** The two rabbits immunized with Sa bacteria had low amounts of IgM antibodies, as demonstrated by ELISA (about 1.2 \(-\log_{10}\)) against LPS Ss before immunization. In sera obtained after primary immunization, IgM and IgG antibody response against Sa bacteria was obtained after 2 days (titer about 3 \(-\log_{10}\) for both classes of antibodies). The preimmune sera of two rabbits immunized with Sb bacteria did not reveal any IgM antibodies against LPS Sb. The primary immunization caused an IgM antibody response after 2 days, whereas IgG antibodies were not revealed until after about 3 weeks (Fig. 2). The IgM titers against LPS did not increase any further after the booster dose and during the hyperimmunization course, whereas the IgG antibodies increased rapidly to values between 6 and 7 \(-\log_{10}\) (Fig. 2). In addition, the CF and BC titers are given for comparison. The sensitivity of the CF and BC methods was about 50- and 150-fold lower than that of ELISA in serum samples taken after primary immunization and hyperimmunization, respectively.

**DISCUSSION**

The present report evaluates the suitability of ELISA for quantitation of antibodies against the CPS and LPS antigens of *H. influenzae* in rabbit antisera.

The investigation showed that the capsular substance of *H. influenzae* types a and b (CPS Ma and CPS Mb) adsorbed to the plastic tubes in sufficient amounts to allow determination of antibodies. Two preparations of purified *H. influenzae* capsular antigen were utilized, one obtained by Sephadex G-150 filtration and diethylaminoethyl Sepharose chromatography (method I) and the other obtained by cetyltrimethylammonium bromide precipitation and Sepharose-2B filtration (method II). They were found to differ in binding capacity to the plastic surface, with CPS II being attached more easily; the optimal concentration was markedly lower, and for CPS Mb II, the necessary attachment time was considerably shorter than for CPS Mb I. Different lots of CPS II preparations showed the same high attachment ability. It was further observed in inhibition studies that the immunological reactivity of CPS Mb II antigen was about 10-fold higher than that of CPS Mb I. In addition, its purity (e.g., low protein content) is a further advantage of method II for preparation of *H. influenzae* capsular antigen for ELISA.

Results obtained by ELISA may be expressed in a variety of ways (15). In this study, the antibody content was expressed as \(-\log_{10}\) of serum dilution representing an endpoint titer. Repeated titration of reference sera showed that there was good reproducibility of results expressed in this way.

For standardization of the assay, the use of reference serum, with known amounts of specific antibodies in all sets of experiments, was necessary to compensate for variation in test parameters such as antigen coating on different occasions, different lots of conjugates, etc. The standard deviation of ELISA described in this study was as low as 0.21 \(-\log_{10}\).

ELISA was found to be a sensitive method for the demonstration of antibodies to *H. influenzae* antigens. By means of this technique, it was possible to measure about 80 ng of IgG antibodies against CPS. However, since IgM antibodies present in the hyperimmune sera may also have contributed to the amount of precipitate obtained at the quantitative precipitation analysis, the sensitivity of ELISA may have been somewhat underestimated. Despite this possibility, the sensitivity was of the same magnitude as that of radioimmunoassays as reported by Norden and Michaels (11). However, other studies have reported higher sensitivity of radioimmunoassay techniques (1). The sensitivity of ELISA with regard to determination of antibodies against LPS was at least 10-fold higher than that of antibodies against CPS. It may be notable that the sensitivity of the assay used in the present study for demonstration of antibodies against *H. influenzae* LPS was about
fourfold higher than that registered in an assay for determination of antibodies against Salmonella O antigen (6).

To obtain high specificity of ELISA, a primary requisite is to use highly purified antigen preparations at the coating of the tubes. The presence of a trace amount of O antigen in the CPS preparation used in ELISA (less than 0.5 μg/ml) should not have influenced the absorbance obtained in the tests performed with this antigen because an absorbance as low as 0.2 was observed with 1 μg of CPS per ml.

ELISA performed with hyperimmune sera against other H. influenzae strains and other species of bacteria revealed antibodies against H. influenzae type a, S. pneumoniae type 6, and E. coli K100 cross-reacting with the CPS Mb II antigen. The capsular antigens of these bacteria are known to have some chemical similarities to the capsular antigen of H. influenzae type b (13, 14).

Using the same experimental parameters in micro-ELISA as in macro-ELISA, no differences in titer values exceeding 0.2 -log10 were recorded. On this basis, the same sensitivity can be assumed for the micro method and macro method.

Although the number of animals included in the study was rather small, some general conclusions concerning the sensitivity of ELISA as compared with that of other serological methods might be drawn.

For estimation of the primary response (IgM class antibodies), ELISA was found to be more sensitive (10- to 100-fold) than the other methods, especially when compared to IHA, which measures capsular antibodies, too.

After the booster dose, in addition to IgM class antibodies, ELISA IgG titers were revealed which were higher than those obtained by other methods.

In the hyperimmune sera to capsulated bacteria, the differences between ELISA titers and the titers of the other methods were less pronounced. In the antisera to noncapsulated bacteria, the higher sensitivity of ELISA was more evident. The discrepancy in sensitivity between BC and ELISA may partly be due to the very high sensitivity of ELISA for the detection of anti-LPS antibodies. Moreover, antibodies against LPS and other cell wall antigens of noncapsulated bacteria are possibly not as effective in the BC assay as are anticapsular antibodies (6a).

ELISA was found to be a suitable method for detection of anticapsular antibodies against H. influenzae type b in humans, too (manuscript in preparation). Especially, its high sensitivity in estimation of primary response antibodies, as observed for the rabbit sera, may be valuable for detection of antibodies during the acute stages of infectious diseases. The micro variant is easier to perform and is more economical with regard to consumption of antigen, conjugate, etc., and this modification can, therefore, be recommended for routine work.

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LITERATURE CITED


