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Immunoglobulin G and M Antibodies to Pneumococcal Polysaccharides Detected by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay has been developed to detect serum immunoglobulin G and M antibodies against pneumococcal polysaccharide antigens. Parameters affecting the specificity and sensitivity of the assay are described. A vigorous antibody response involving both the immunoglobulin G and M classes was demonstrated after pneumococcal polysaccharide immunization in normal adults. Studies with this enzyme-linked immunosorbent assay technique will allow further understanding of the biology of the primary and secondary immune response to *Streptococcus pneumoniae* in normals as well as in those persons most susceptible to infection with that organism.

Streptococcus pneumoniae is a common cause of pneumonia, otitis media, bacteremia, and meningitis throughout the world. In spite of the use of antibiotics, attack rates have remained essentially unchanged. With approximately 400,000 to 500,000 cases reported annually in the United States, *S. pneumoniae* remains the major cause of community-acquired bacterial pneumonia. *S. pneumoniae* annually accounts for 1.5 to 2.5 cases of meningitis per 100,000 population. *S. pneumoniae* is an even more common and more serious pathogen in the elderly and in those with sickle cell disease, anatomical or functional asplenia, immunodeficiency, malignancy, nephrotic syndrome, or chronic liver disease. Even with antibiotic therapy, morbidity and mortality from *S. pneumoniae* infections continue to be a significant problem. The overall case fatality rate for *S. pneumoniae* pneumonia is estimated to be 5 to 10%. This figure is even higher in the elderly and those with chronic disease, where estimates as high as 25% have been reported. The mortality rate approaches 40% in children with *S. pneumoniae* meningitis, and permanent sequelae are noted in at least one-half of the survivors (4, 15).

The recent appearance of some *S. pneumoniae* with multiple antibiotic resistance (3, 7) has, in addition to the above factors, led to a renewed interest in the efficacy of immunization with polyvalent pneumococcal polysaccharide (PPS) vaccine. Currently, there are two methods in use to measure the antibody response to pneumococcal polysaccharide antigens: radioimmunoassay (G. Schiffman and R. Austrian, Fed. Proc. **30**:658) and indirect hemagglutination (2). Although each of these methods is sensitive and specific, they both measure total antibody produced and do not differentiate between immunoglobulin G (IgG) and IgM classes of antibody unless the serum is fractionated before assay. To more completely understand the biology of the immune response to PPS, an assay which detects immunoglobulin class-specific antibody is needed.

Enzyme-linked immunosorbent assay (ELISA) has recently been used to measure the serological response to a variety of infectious agents (8, 16, 19). ELISA techniques are reported to be more sensitive than agglutination, quantitative precipitation, passive hemagglutination, immunofluorescence, and complement fixation (19). The sensitivity of ELISA has equalled that of radioimmunoassay (8). The specificity of ELISA for antibody detection can be high and is determined by the quality of the antigen used in the system (19). Furthermore, these techniques offer the potential of detecting immunoglobulin class-specific antibodies in unfractionated sera (i.e., IgG and IgM) (10, 11, 12, 14, 18), allowing detailed investigation of primary and secondary antibody responses. ELISA also has the advantages of using inexpensive reagents with long shelf lives, lack of radiation hazard, or need for expensive equipment for the detection of radioactivity, and relatively simple manipulations with a potential for automation. In this paper, we describe an ELISA technique for the detection of IgG and IgM class-specific antibody to individual serotypes of pneumococcal polysaccharide.

MATERIALS AND METHODS

Preparation of enzyme-conjugated anti-immunoglobulins. The IgG fraction of rabbit anti-human IgG antisera (gamma-chain specific; Cappel Lab-

oratories) was coupled to alkaline phosphatase by the Engvall and Pearlmann modification (10) of the method of Avrameas (5). Alkaline phosphatase (Sigma, type VII; specific activity greater than 1,000 U/mg of protein) was centrifuged at 1,000 rpm for 10 min and the clear supernatant was discarded. The remaining enzyme-protein precipitate was added to the rabbit anti-human IgG in a final ratio of 3.0 mg of enzyme per mg of anti-immunoglobulin. After overnight dialysis against three changes of phosphate-buffered saline, glutaraldehyde was added to a final concentration of 0.2%. This coupling reaction was allowed to proceed for 2 h at room temperature. The mixture was diluted to 1.0 ml with phosphate-buffered saline and dialyzed overnight against three changes of phosphate-buffered saline. An analogous procedure was used to conjugate alkaline phosphatase to rabbit anti-human IgM (mu-chain specific; Cappel Laboratories).

The activity and specificity of these enzyme-linked anti-immunoglobulins were tested by reacting each with varying concentrations of chromatographically purified human IgG and IgM (Cappel Laboratories) adsorbed onto a flat-bottomed polystyrene microtiter plate (Linbro Chemical Co.). A series of preliminary experiments were performed to determine the optimal method of washing the microtiter plates after the addition of enzyme-labeled anti-immunoglobulin. Efficient removal of the unbound conjugate was accomplished with three separate distilled water washes followed by a 10-min soak in phosphate-buffered saline containing 0.05% polysorbate (Tween 20) as a wetting agent. After washing, the amount of enzyme-labeled anti-immunoglobulin bound to the immunoglobulin-coated plates was determined by reaction with enzyme substrate as described below.

Preparation of antigen-coated plates. The method used was modified from that of Voller et al. (17). The optimal dilution of the reagents was determined by checkerboard titration. A 50-μl sample of a single type-specific rabbit anti-PPS antiserum (Laboratory of the State Department of Health, Albany, N.Y.), diluted in normal saline, was added to each well of a microtiter plate. Passive adsorption of the antiserum was accomplished by incubation of the plate at 4°C for 18 h. The plate was then washed as above. A 50-μl sample of purified PPS (of the type appropriate to the antiserum coating the plate) (Merck Sharp & Dohme) diluted in 0.05 M sodium carbonate buffer (pH 9.8) was added to each well, and the plate was then incubated at 37°C for 2 h. After additional washes, the plate was ready for use in the ELISA. Separate plates, each coated with a single PPS type, were prepared for each of the PPS types (III, VIII, XVIII) tested. Control plates to test for cross-reactivity between the type-specific rabbit anti-PPS antiserum and the various PPS types, as well as controls for the purity of the PPS antigens used, were prepared by adsorbing with type-specific rabbit anti-serum and then reacting those plates with PPS of a different serotype.

ELISA procedure. The human sera to be tested were diluted serially in 0.5 M NaCl containing 0.5% Tween 20 (pH 8.3) and added in 50-μl amounts to each well of the antigen-coated plates. Optimal time of incubation of the human sera was determined in separate experiments. After incubation, the plates were washed and 50 μl of the appropriate enzyme-linked anti-immunoglobulin was added and allowed to react at 37°C. The kinetics of this reaction were also determined in separate experiments. The plates were again washed, and 50 μl of *p*-nitrophenyl phosphate substrate (1.0 mg/ml) was added (Sigma 104; diluted in 0.05 M sodium carbonate buffer, pH 9.8, containing 1 mM magnesium chloride). After incubation at room temperature, absorbance of the yellow color produced by the action of the enzyme (now bound to the solid phase) on the substrate was measured at 405 nm in a colorimeter (Titertek Multiskan, Flow Laboratories) which reads through the bottom of the microtiter plates. The appropriate time of incubation for the substrate reaction was determined in separate experiments. If the measurement was delayed beyond 2 h, 25 μl of 3.0 M NaOH was added to each well to stop the reaction.

To control for the nonspecific adherence of immunoglobulin contained in the test serum, each serum was also serially diluted in wells to which the type-specific rabbit anti-PPS antiserum alone had been adsorbed (i.e., wells lacking the PPS antigen). The results of the ELISA were expressed as the endpoint titration defined as the highest dilution of serum producing an optical density in the test wells (i.e., rabbit anti-PPS + antigen) which was twice that of the same dilution in the control wells (i.e., rabbit anti-PPS alone) (20).

2-Mercaptoethanol inactivation of test sera. As a test of the specificity of the ELISA for measuring IgM, sera were tested in the system before and after inactivation with 2-mercaptoethanol (2). 2-Mercaptoethanol (0.2 M) was added in equal volumes to a portion of the sera to be tested and incubated for 1 h at 37°C. Antibody titers were performed on these samples as well as portions diluted with equal volumes of 0.5 M NaCl containing 0.5% Tween 20.

Serum samples. Eight healthy adult controls (employees of the hospital and research laboratories of the University of California, San Francisco) volunteered to receive the polyvalent pneumococcal vaccine (Pneumovax, Merck Sharpe & Dohme). A 0.5-ml amount of the vaccine was administered subcutaneously, providing 50 μg of each polysaccharide type (types 1, 2, 3, 4, 6, 8, 9, 12, 14, 19, 23, 25, 51, and 56). Preimmunization and 2-week postimmunization sera were obtained and frozen at −70°C until use. Each individual had a positive antibody response to immunization as detected by indirect hemagglutination (2). Serum samples from three individuals with acquired hypogammaglobulinemia were obtained as negative controls. Cord blood samples were assayed as IgM-negative controls. Antibody detected in the cord blood would have to be due to passive transfer and hence would be primarily of the IgG class. Immunoglobulins were assayed by radial immunodiffusion (Quantiplate; Kallestad Laboratories).

RESULTS

Sensitivity and specificity of the enzyme-labeled anti-immunoglobulins. The sensitiv-

ity of the enzyme-labeled anti-immunoglobulins was determined by incubation of these reagents with plates coated with varying dilutions of purified IgG or IgM. As shown in Fig. 1, increasing the concentration of the enzyme-labeled anti-IgG markedly increased the sensitivity of the assay. Similar results were obtained in testing the enzyme-labeled anti-IgM. The specificity of these reagents was determined by separately reacting each with plates coated with varying concentrations of either IgG or IgM. The results shown in Fig. 2 demonstrate that the enzyme-labeled anti-human IgG at a 1:100 dilution is capable of detecting approximately 10 ng of IgG per ml. No cross-reactivity for IgM was apparent. Similar results were obtained in experiments with the enzyme-labeled anti-human IgM at a 1:75 dilution, and no cross-reactivity for IgG was noted.

Optimal antigen coating of plates. Attempts to adsorb polysaccharide-containing antigens to plastic surfaces have often failed. To overcome this problem, we used a modification of the method of Voller et al. (17) whereby antigen-specific rabbit antibody is first adsorbed passively to the plates. The antigen is subsequently reacted with that rabbit antibody, thus forming a stable antigen(-antibody)-coated solid surface. To determine the appropriate concentrations of the reagents, varying dilutions of type-specific rabbit anti-PPS and varying concentrations of the homologous purified PPS antigen were added to a plate in checkerboard titration. The plates were then reacted with a 1:4 dilution of known positive immune human serum followed by incubation with enzyme-labeled rabbit anti-human IgG and finally, enzyme substrate. As shown in Fig. 3, optimal coating of the microtiter plates occurred with dilutions of rabbit anti-PPS III between 1:1,000 and 1:10,000. Antigen coating was also found to be maximal with a PPS III antigen concentration of 0.01 mg/ml. At higher concentrations of antigen, there was a decrease in absorbance. The reaction of specific PPS antigen with the antibody-coated plate occurs quickly. Little or no difference was noted when PPS was incubated with its antibody-coated plate for 2 h or for 24 h (data not shown). Similar results were obtained in titrations for PPS VIII and PPS XVIII. In separate experiments, no cross-reactivity was found between these lots of type-specific rabbit anti-PPS and the nonhomologous purified antigens (data not shown).

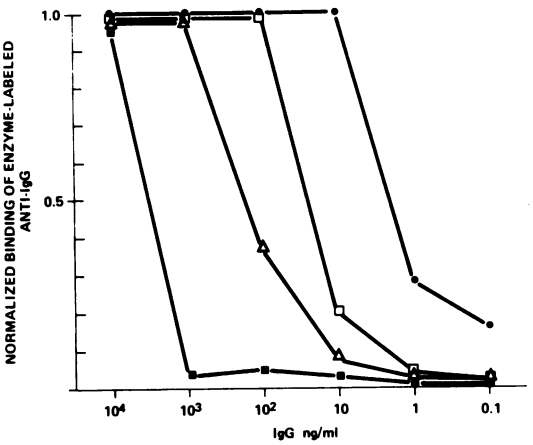

FIG. 1. *Effect of concentration of enzyme-labeled anti-IgG on ELISA sensitivity. Varying concentrations of human IgG were adsorbed to the wells of microtiter plates. Dilutions (●, 1:10^2; □, 1:10^3; △, 1:10^4; ■, 1:10^5) of enzyme-labeled anti-IgG were incubated in the IgG-coated wells for 1 h. After washing, p-nitrophenyl phosphate substrate was added and incubated and the optical density (OD) at 450 nm was measured. Data points were normalized by dividing the measured OD for each IgG concentration (minus background) by the maximum OD (minus background). Maximum OD obtained for each dilution of enzyme-labeled anti-IgG was the absorbance measured in wells coated with 10^4 ng of IgG per ml: normalized absorbance = (measured OD − background OD)/(maximum OD − background OD). Increasing the concentration of the enzyme-labeled anti-IgG led to increased sensitivity in ELISA. Similar results were obtained in testing the enzyme-labeled anti-IgM on IgM-coated plates.*

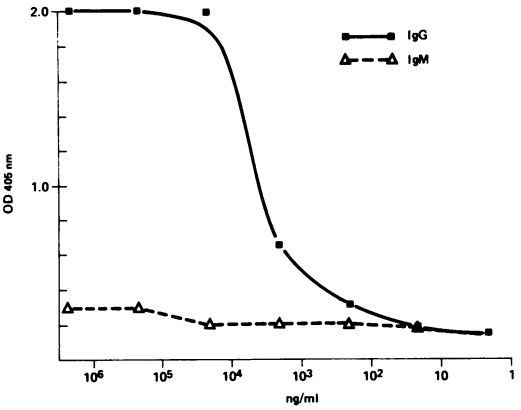

FIG. 2. *Specificity of enzyme-labeled anti-IgG. Varying concentrations of human IgG or IgM were adsorbed to the wells of microtiter plates. A 1:100 dilution of enzyme-labeled anti-IgG was then incubated in each well. Plates were then washed, p-nitrophenyl phosphate substrate was added, and optical density (OD) at 450 nm was measured. There was no cross-reactivity between the anti-IgG and IgM. Similar results were obtained in specificity tests of the enzyme-labeled anti-IgM showing no cross-reactivity between the anti-IgM and IgG.*

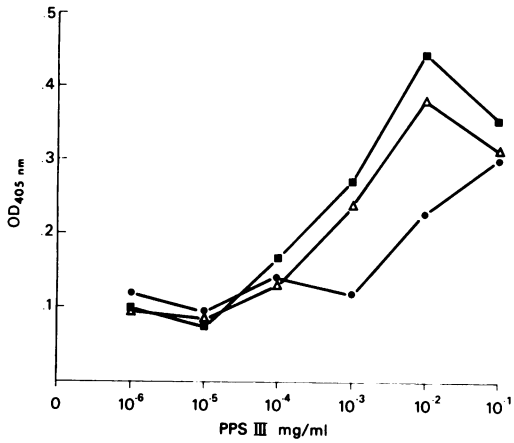

FIG. 3. *Optimal antigen-coating of plates. PPS III antigen-coated plates were prepared by incubating varying dilutions of rabbit anti-PPS III (■, 1:10³; △, 1:10⁴; ●, 1:10⁵) and then varying concentrations of PPS III antigen in a checkerboard titration. The plates were then reacted with a 1:4 dilution of human serum known to contain anti-PPS III antibody (determined previously by indirect hemagglutination) followed by incubation with enzyme-labeled anti-IgG, and finally, enzyme substrate. Optimal antigen-coating occurred with dilutions of rabbit anti-PPS III of 1:10³ to 1:10⁴, and with a PPS III antigen concentration of 0.01 mg/ml. Similar results were obtained in experiments to determine conditions for maximum antigen coating with PPS III and PPS XVIII.*

Kinetics of binding of human serum antibody. The rate of binding of IgG and IgM antibodies in human serum was determined by using plates coated with a 1:1,000 dilution of rabbit anti-PPS III and subsequently reacted with PPS III (0.01 mg/ml). Known positive immune human serum was added to the plates in serial twofold dilutions. Separate experiments for both IgG and IgM antibody binding were performed to determine whether the kinetics of antibody binding was dependent on immunoglobulin class. The plates were allowed to react with the immune serum for varying periods of time, washed, and reacted with the appropriate enzyme-labeled anti-immunoglobulins and washed again, and then substrate was added. The results, shown in Fig. 4, demonstrate that maximum binding of human anti-PPS III antibody occurs with 30 min of incubation on the plate. Thereafter, there is a decrease in the amount of antibody fixed to the solid phase. Similar kinetics were observed for the binding of both IgG and IgM antibodies.

Kinetics of enzyme-labeled anti-immunoglobulin binding. By reacting each of the enzyme-labeled anti-immunoglobulins for varying periods of time with plates coated with antigen and known antibody-positive human serum, the optimal time of conjugate incubation was determined. Maximum binding occurred at 3 h in both the IgG and IgM antibody assays (Fig. 5).

Kinetics of substrate reaction. Experiments to determine the optimal time of incubation of the *p*-nitrophenyl phosphate substrate demonstrated that 1 to 4 h allowed detection of 1.0 ng of IgG per ml (Fig. 6). Shorter incubation times were found to decrease the sensitivity of

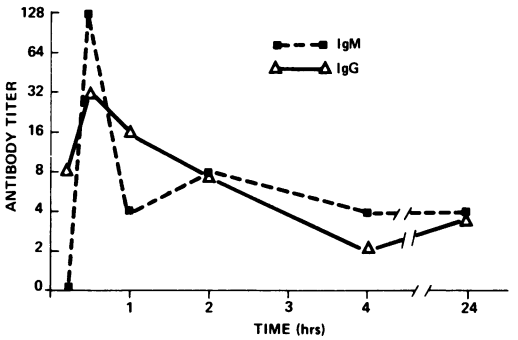

FIG. 4. *Kinetics of binding of human serum antibody. PPS III antigen-coated plates were prepared as described and known positive immune human serum (determined by indirect hemagglutination) was added in serial twofold dilutions. The plates were allowed to react with the immune serum for varying periods of time. After washing, the plates were reacted with the enzyme-labeled anti-immunoglobulins and finally substrate to detect IgG (△) and IgM (■) antibodies. Maximum binding of both IgG and IgM anti-PPS III antibodies occurred at 30 min of incubation.*

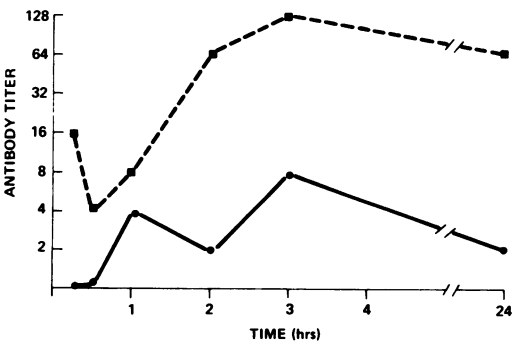

FIG. 5. *Kinetics of enzyme-labeled anti-immunoglobulin binding. PPS III antigen-coated plates were prepared as described and serial twofold dilutions of known positive immune human serum (determined by indirect hemagglutination) were incubated for 1 h. After washing, the plates were reacted with enzyme-labeled anti-IgG or anti-IgM for varying periods of time. Maximum binding occurred at 3 hours for both IgG (●) and IgM (■) antibodies.*

the assay, and longer incubation times were found inconvenient for performing the assay in a single day.

Class-specific antibody response to pneumococcal polysaccharide immunization in normal adults. A vigorous antibody response involving both the IgG and IgM classes was found in serum samples obtained 2 weeks after immunization with a polyvalent pneumococcal polysaccharide vaccine in eight normal adults (Fig. 7). Despite variability between individuals in the mean preimmunization IgG antibody titers to the three types of PPS tested, a fourfold or greater rise in the titer of IgG antibody to all three types was demonstrated. High titers of preimmunization IgG antibody, as seen with type XVIII, did not preclude a significant antibody increase after immunization.

Low (less than or equal to 2) mean preimmunization IgM antibody titers were found with all three types of PPS tested. Large increases in IgM antibody titers were noted after immunization with all types (Fig. 7). Also, higher mean post-immunization IgM antibody responses occurred when mean preimmunization IgG titers were low (i.e., with PPS III).

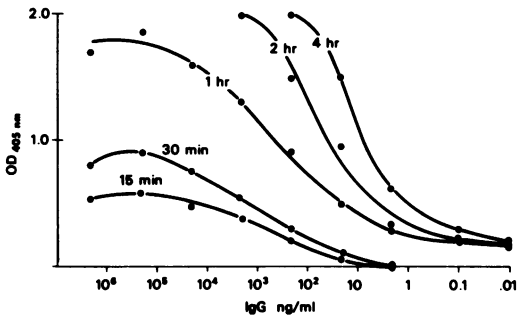

FIG. 6. *Kinetics of substrate reaction. Varying concentrations of human IgG were adsorbed to the wells of microtiter plates. A 1:100 dilution of enzyme-labeled anti-IgG was then incubated in the wells for 1 h, the plates were washed, and 50 μl of 1.0-mg/ml p-nitrophenyl phosphate substrate was added. After varying times of incubation (shown in figure) 25 μl of 3.0 M NaOH was added to each well to stop the substrate reaction, and optical density (OD) at 405 nm was measured. Longer substrate reaction times enhanced the sensitivity of the assay.*

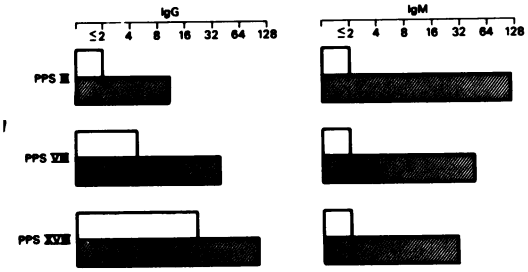

FIG. 7. *Mean antibody titers in normal adults. Symbols: □, preimmunization; ▨, postimmunization.*

Sera from three patients with acquired hypogammaglobulinemia served as known negative controls. No IgG or IgM antibody to PPS III was detected in these sera (Table 1). Three cord blood sera were tested as possible sources of "pure" IgG antibody (essentially free of IgM due to lack of maternal transfer of that immunoglobulin class). As shown in Table 1, none of the sera had detectable IgM antibody levels; however, two of the three had measurable levels of IgG antibody to PPS III.

2-Mercaptoethanol inactivation of antibody in three normal's sera (Table 2) resulted in a decrease to undetectable levels in the titer of IgM antibody.

Reproducibility of the method was insured by performing replicate assays of a single serum sample. When performed on the same day, the variability was within one dilution; performed on different days the variability was within two dilutions. To minimize variation, pre- and postimmunization samples from a given individual were always run at the same time on the same microtiter plate.

TABLE 1. *Detection of IgG and IgM antibody to PPS III in sera*

Serum	ELISA antibody titers[a]		Serum immunoglobulins (mg/dl)	
	IgG	IgM	IgG	IgM
Hypogammaglobulinemia patient				
1	<1:2	<1:2	6	1
2	<1:2	<1:2	225	6
3	<1:2	<1:2	86	5
Cord blood sample				
4	1:2	<1:2	1,680	8
5	1:8	<1:2	1,970	11
6	>1:512	<1:2	1,260	24

[a] Antibody to PPS III.

TABLE 2. *Effect of 2-mercaptoethanol inactivation of antibody in normal sera*

Subject	ELISA antibody titers[a]			
	IgG		IgM	
	Before 2-ME[b]	After 2-ME	Before 2-ME	After 2-ME
A	<1:2	<1:2	>1:512	<1:2
B	<1:2	<1:2	1:64	<1:2
C	1:4	1:2	1:8	<1:2

[a] Antibody to PPS III.
[b] 2-ME, 2-Mercaptoethanol.

DISCUSSION

ELISA techniques have been used to detect antibody to a variety of antigens, haptens, and antibodies (reviewed in references 8, 16, 19). Since the original description of this type of assay by Engvall and Perlmann in 1971 (10), ELISA has proven to be a sensitive, specific, efficient, and objective technique for detecting antibodies to a variety of protein and lipopolysaccharide antigens. In this paper, we describe an ELISA system capable of detecting IgG and IgM class-specific antibodies to individual PPS types. To enhance sensitivity and minimize variability, a number of parameters affecting the assay were examined.

In preparation of antigen-coated solid surfaces, polysaccharide antigens have been found to attach poorly to polystyrene. To insure optimal binding of the PPS antigen, we first prepared the wells of polystyrene microtiter plates by adsorbing to them type-specific rabbit anti-PPS antiserum. Subsequent binding of purified individual types of PPS antigens to the homologous antibody-coated plates formed a stable antigen-coated solid phase. Specificity of the rabbit anti-PPS antisera and purity of the individual PPS antigens were ensured by incubation of rabbit antisera-coated plates with each nonhomologous antigen.

The optimal dilution of type-specific rabbit anti-PPS and the optimal concentration of PPS antigens were determined by checkerboard titrations. With very dilute rabbit antisera, less than optimal antigen binding occurred. With large concentrations of PPS, a prozone effect was observed (Fig. 3). Similar decreases in binding with excessive concentrations of antigen in the coating solution have been noted by others (9, 13). This phenomenon could be due to elution of some of the antigen during the subsequent reaction with human serum antibody. The excess antigen that was not tightly bound to the plate could bind to the human antibody in solution. This binding would decrease the amount of human antibody free to react with the antigen that remained tightly bound to the solid phase (13). Alternatively, high concentrations of antigen could be responsible for elution of some of the passively adhered rabbit anti-PPS during the antigen coating step, resulting in less total antigen adhering to the plate. Again, less human serum antibody would then be bound to the solid phase.

Once the rabbit anti-PPS was adsorbed to the plate, the subsequent binding of homologous PPS antigens occurred quickly. Little difference was found in the assay performed after 2 or 24 h of antigen coating time. We confirmed the specificity of these reagents in separate experiments where no cross-reactivity was found between the type-specific rabbit anti-PPS antisera and the nonhomologous antigens. These controls are crucial and must be repeated with each new lot of antigen and antibody prepared.

The sensitivity of the ELISA was determined, in part, by the length of time that the human serum was allowed to react with the antigen-coated plate (Fig. 4). Maximum binding occurred at 30 min. Longer incubation times were associated with decreased antibody titers, probably due to elution of antigen from the solid phase.

Sensitivity in the assay is also dependent on the concentration and the time of incubation of enzyme-labeled anti-immunoglobulin (Fig. 1 and 5). Excessive concentrations of this reagent lead to high nonspecific adherence to the wells, and concentrations too low lead to decreased sensitivity of the assay. The optimal concentration of enzyme-labeled anti-immunoglobulin must be determined for the system each time this reagent is prepared. As shown in Fig. 5, the time of incubation of this conjugate is also important in maximizing detection of antibody.

The kinetics of the substrate reaction can also affect the sensitivity of the ELISA. As shown in Fig. 6, longer incubations of the *p*-nitrophenyl phosphate substrate increased the sensitivity of the assay without appreciably increasing the background color. For convenience in completing the assay in a single day, we chose substrate incubation times of 1 to 2 h.

Results of antibody detection in the present system were expressed as endpoint titration. It has been shown by Ahlstedt et al. (1) and Butler et al. (6) that some ELISA procedures, in spite of being highly sensitive, detect high-avidity antibody in preference to low-avidity antibody. The total quantity of antibody (as measured by quantitative double antibody precipitation) may not be measured, therefore, in some ELISA systems. To test whether a similar phenomenon was occurring with our ELISA for antibody to PPS, purified immunoglobulin class-specific PPS type-specific antibodies could be measured in a gravimetric determination of total antibody (i.e., precipitation in radioimmunoassay), and the results could be correlated with the endpoint titrations in ELISA. Such studies are currently in progress. As noted by Keren (13), however, measuring high-avidity antibody may be more important than measuring total antibody in evaluating the effectiveness of bacterial vaccines, since low-avidity antibodies in some systems are less protective against antigenic challenge than are antibodies of high avidity.

The results of antibody determination on pre- and postimmunization sera from normals (Fig. 7), on sera from hypogammaglobulinemic patients, and on cord blood (Tables 1 and 2) clearly indicate that the present ELISA method can distinguish IgG versus IgM antibody in the same unfractionated serum sample. This capability, which is not present in indirect hemagglutination and radioimmunoassay for PPS antibody, will permit investigation of the primary and secondary antibody response to PPS.

In summary, we have described an ELISA system which is sensitive, reproducible, and specific for individual serotypes of PPS antigen. In contrast to other assays of antibody to PPS, ELISA is capable of efficiently detecting IgG and IgM classes of antibody. Studies with this technique will allow further understanding of the biology of the primary and secondary immune response to the pneumococcus in normal persons as well as in those persons most susceptible to infection with that organism.

ACKNOWLEDGMENTS

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