

## Human-Isotype-Specific Enzyme Immunoassay for Antibodies to Pneumococcal Polysaccharides

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**A simple enzyme immunoassay has been developed to allow the quantitation of the human response to immunization with pneumococcal polysaccharide. The assay uses the 14-valent vaccine (Pneumovax) as a convenient antigen to adsorb to the solid-phase microdilution plate wells and commercially available isotype-specific antibody conjugates. The results have been expressed as arbitrary pneumococcal polysaccharide antibody units by reading off a standard curve constructed by using heterogeneous pooled serum. All nonimmunized subjects tested had immunoglobulin G (IgG) antibodies present in serum. All six control subjects who were immunized with Pneumovax demonstrated an IgG response, and the majority responded with a rise in IgA- and IgM-specific antibody concentrations at a mean of 6 weeks postimmunization. Five out of six cord sera tested contained IgG antibodies only, which were present in concentrations similar to those seen in adults, whereas in 6- to 12-month-old infants only low levels of IgG and IgM and no IgA antibodies were detected. Serum taken from 10 hypogammaglobulinemic patients immediately prior to infusion of immunoglobulin showed low to negative IgG antibody concentrations, and no IgA or IgM antibody was present.**

The majority of studies of the antibody response to pneumococcal polysaccharide (PNPS) vaccination have been performed by radioimmunoassay (12), which is designed to measure the quantity of precipitable antibody, irrespective of the immunoglobulin class. Estimates of the protective concentration of anti-PNPS antibody in humans have been formed on the basis of results obtained by the above method (16).

Enzyme-linked immunoassay (EIA) has several advantages over radioimmunoassay, including greater reagent stability, greater ease of use with avoidance of the need for gamma counting, and greater adaptability for measuring isotype-specific antibody responses (8). EIAs are now being used more extensively for detection of PNPS antibodies (1, 4, 5, 17, 19, 20).

Different immunoglobulin classes have different opsonic capacities (3). Giebink et al. (9) have shown that splenectomized, as well as nonsplenectomized, children may show an increase in total serum antibody, as measured by radioimmunoassay, without showing enhanced opsonic activity after PNPS vaccination. To more completely understand the biology of the immune response to PNPS, an assay which detects immunoglobulin class-specific antibody is needed.

In developing this EIA, we have used a 14-valent pneumococcal vaccine (Pneumovax; Merck Sharp & Dohme Pty. Ltd.) as the solid-phase antigen. This provided a convenient means of determining the isotype-specific response to immunization.

### MATERIALS AND METHODS

**Serum samples.** Serum samples were collected from the following groups and stored at  $-70^{\circ}\text{C}$  until assayed: (i) 14 laboratory personnel (these sera representing nonimmunized control adults [aged 22.7 to 46.9 years]); (ii) 6 healthy adult volunteers, aged 24.5 to 44.9 years (mean, 28.5 years), who received Pneumovax, a polyvalent pneumococcal vaccine containing 50  $\mu\text{g}$  of capsular polysaccharide from each of 14 pneumococcal serotypes (serum samples were collected

before immunization and at 4 to 8 weeks postimmunization, with an average postimmunization period of 6 weeks); (iii) 10 hypogammaglobulinemic patients receiving four weekly infusions of intravenous gamma globulin (sera were collected immediately prior to infusion); (iv) 6 normal babies, aged 6 to 12 months; (v) 6 cord blood samples. As well as the above, the Australian reference serum ASPS 78/1 (22) and a 1:10 dilution of normal human gamma globulin (Commonwealth Serum Laboratories) were assayed for PNPS antibody.

**EIA.** The sera were assayed by EIA using a modification of the methods of Melville-Smith and Sheffield (17). The tests were carried out in disposable 96-well flat-bottom (Inter Med) Nunc Immunol plates. Pneumovax was dialyzed against 0.05 M carbonate buffer (pH 9.8). The optimal binding concentration was obtained by checkerboard titration. A final concentration of 3.5  $\mu\text{g}/\text{ml}$  was used in 100  $\mu\text{l}$  of 0.05 M carbonate buffer (pH 9.8) to coat the appropriate wells not on the edge of the plate. After an initial incubation at  $37^{\circ}\text{C}$  for 3 h, the coated plates were left at  $4^{\circ}\text{C}$  overnight. All incubations were carried out in sealed moist chambers. At the completion of incubation, the wells were washed for 15 s with 0.05 M phosphate-buffered saline (PBS) (pH 7.2) containing 0.05% Tween 20 (PBS-Tween) by using an automatic microdilution plate washer (Immunowash-12 machine; Medos Company Pty. Ltd.). Appropriate dilutions of serum samples were added in 50- $\mu\text{l}$  amounts and incubated for 1 h at  $37^{\circ}\text{C}$ . The diluent used was 0.05 M PBS (pH 7.2) with 1% bovine serum albumin (BSA, fraction V; Commonwealth Serum Laboratories) (PBS-BSA) to reduce nonspecific binding.

After incubation, the wells were washed as above and drained. The appropriate wells were then incubated with 50  $\mu\text{l}$  of optimal dilutions (in PBS-BSA) of affinity-purified goat anti-human immunoglobulin G (IgG) (1:2,000), IgA (1:1,000), or IgM (1:1,000) conjugated to alkaline phosphatase (immunodiagnostic reagents from Tago, Inc.). Incubations were performed for 1 h at  $37^{\circ}\text{C}$ , and the wells were washed as before. The substrate used was *p*-nitrophenyl phosphate (alkaline phosphatase test kit; Boehringer Mannheim), which was diluted to 1 mg/ml in 10% diethanolamine buffer

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with  $MgCl_2$  (pH 9.8). Substrate solution (50  $\mu$ l) was added to each test well and incubated for 30 min at 37°C in light-sealed containers. The reaction was quenched by the addition of 25  $\mu$ l of 5 N NaOH, and the optical density was read at 405 nm by using an automated Microelisa reader (Flow Titertek, Multiscan; Flow Laboratories). The optimal times and temperatures for all the above steps were determined in separate experiments by using paired pre- and postvaccination sera.

**Standard sera and controls.** The first row of wells in every microdilution plate received buffer in each step of the assay. The same wells also received substrate and reaction-quenching solution. These wells were used as blanks to zero the Microelisa reader. Background absorbances were calculated from control wells on each plate with no conjugate and no sera. These absorbance readings were generally less than 0.07. The background absorbance reading was subtracted from standard, control, and test sample absorbance values for the calculation of PNPS antibody unit values.

The following sera were included in each Microelisa plate. (i) For construction of the standard curve and assigning of PNPS antibody unit values, three different sera were used for each of the IgG, IgA, and IgM pneumococcal antibody-detecting EIAs. The standards were made from a pool of sera from immunized and nonimmunized adult volunteers. The starting dilution of the sera used for standard curve construction was assigned an arbitrary value of 1,000 U/ml. (ii) A cord blood sample with no detectable (<2 U/ml) IgG, IgA, or IgM antibodies to pneumococcal polysaccharide was used as a negative control serum. (iii) Three different sera, A, B, and C, covering three points on the standard curve, were used in duplicate in each of the assays for IgG, IgA, and IgM antibodies. The antibody units of the above sera had been previously determined from the mean absorbance value of 20 assays each, read against the standard curve. The allotted values of positive internal control sera (in PNPS antibody units per ml) were as follows: 500 for serum A, 62.5 for serum B, and 2 for serum C.

**Reproducibility of assays.** The positive internal control sera A, B, and C were tested 15 times each during one assay to assess intrarun variation. This was repeated on two different days. To determine the interrun variation of the assay, the internal control sera A, B, and C were tested repeatedly in assays over many months.

To minimize variation, all of the serum samples, controls, and standards were assayed in duplicate and in a pattern described previously (21). The absorbance values of duplicates were averaged, and the unit values were read from the standard curve. If the absorbance values for a sample did not fall within the linear part of the curve, the test was repeated using a different dilution of the serum.

The assay results were accepted only if (i) the PNPS antibody unit values of the internal controls fell within the interrun variation, (ii) the unit value of the negative control serum was less than 2 U/ml, and (iii) the background absorbance was less than 0.07.

**Liquid-phase absorption studies.** Six paired pre- and post-immunization sera with a fourfold or greater rise in PNPS antibody after immunization, as determined by EIA, were used.

The above serum samples at a dilution of 1:80 were added to equal volumes of a 1:10 dilution of PNPS or buffer (PBS-BSA). The mixtures were incubated at 37°C for 30 min and were left sealed overnight at 4°C. Both unabsorbed and absorbed sera were then assayed simultaneously in duplicate by EIA for IgG PNPS antibodies.

**Serum IgG quantitation.** A Technicon Auto Analyzer II

Fluoronephelometer was used to quantitate IgG levels (7) in the pre- and postimmunization samples used in the absorption studies.

## RESULTS

Titration curves for determination of the optimal concentration of PNPS for precoating are given in Fig. 1. The concentration of PNPS chosen for further assays was 3.5  $\mu$ g/ml.

PBS-BSA was chosen as the diluent for sample and conjugate dilution steps after comparison of PBS alone, PBS-BSA, and PBS with 0.1% gelatin. Each diluent gave similar absorbance readings with a positive reference serum, but unacceptably high readings were seen with serum from a hypogammaglobulinemic patient in the presence of PBS and PBS-gelatin.

The effect of liquid-phase absorption of serum at a 1:80 dilution with an equal volume of a 1:10 dilution of PNPS or PBS-BSA on the absorbance readings obtained in the IgG EIA are shown in Fig. 2. A decrease in absorbance values is seen in the presence of PNPS in prevaccination serum and in immune serum, demonstrating assay specificity for PNPS.

The lower limit of detection was represented by two-time background readings for IgG, IgA, and IgM antibody to PNPS and was approximately 2 U/ml for each isotype.

The intrarun coefficient of variation for the assay, as determined by the three internal controls (A, B, and C) for IgG, IgA, and IgM PNPS antibody, was 14.1, 17.8, and 19.5%, respectively. The interrun coefficient of variation was 20.5% for IgG, 21.0% for IgA, and 20.9% for IgM.

The 14 nonimmunized adult controls had medium titers of PNPS of  $50.9 \times 10^3$  U/ml for IgG (range,  $16.6 \times 10^3$  to  $98.3 \times 10^3$  U/ml), <10 U/ml for IgA, and <10 U/ml for IgM. None of the sera from the 10 hypogammaglobulinemic patients had measurable IgA and IgM antibodies, and IgG antibody concentrations in all 10 were less than 10 U/ml. All but one of the six cord blood sera tested had IgG antibody levels in the range of the adult controls. The sera from the six infants aged 6 to 12 months had IgG and IgM antibodies at less than 10 U/ml, and no detectable IgA antibodies. Isotype-specific antibody concentrations in arbitrary units per ml for pre- and postimmunization sera for the six immunized adult volunteers are given in Table 1. All volunteers had a threefold or greater rise in IgG antibody after immunization, four had a twofold or greater rise in IgA antibody, and three had a twofold or greater rise in IgM antibody.

## DISCUSSION

The virulence of encapsulated *Streptococcus pneumoniae* is attributed to the antiphagocytic capacities of the PNPS capsule. The development of specific anticapsular antibodies is the aim of immunization with purified PNPS (13, 23).

The EIA described in this report has been used to detect the human isotype response to PNPS. The assay is simple, reproducible, and specific and offers an opportunity to obtain additional information with respect to the immune response to pneumococcal vaccine.

The response to Pneumovax not only represents the response to type-specific polysaccharides but also the response to the pneumococcal C polysaccharide which the vaccine contains (14). Similarly the "resting" or preimmunization specimens almost certainly contain antibodies to the C polysaccharides, as well as to a variable number of the type-specific polysaccharides.

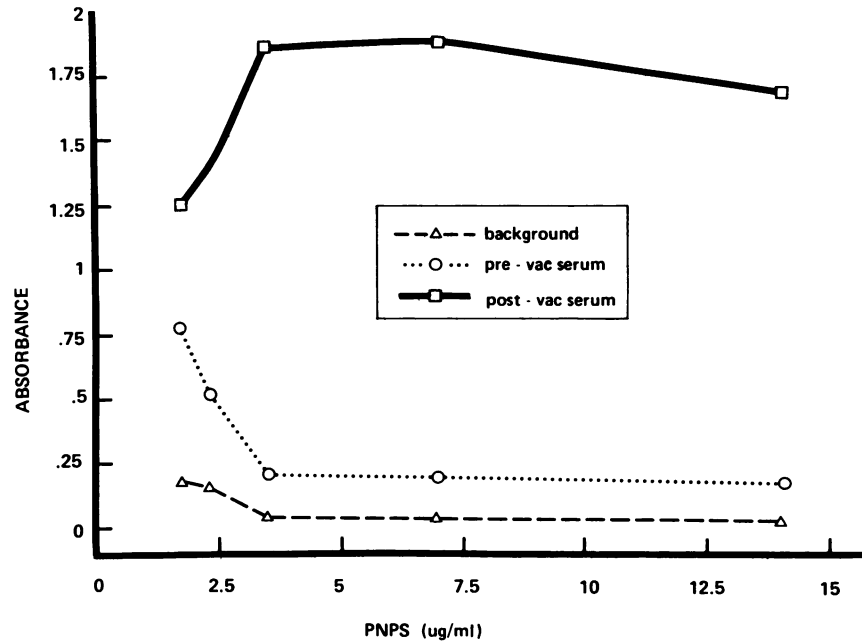


FIG. 1. To determine the optimal PNPS concentration, a preimmunization (pre-vac) serum and corresponding postimmunization (post-vac) serum were assayed at various concentrations of PNPS precoat solution. A concentration of 3.5  $\mu\text{g/ml}$  was chosen as the optimal concentration of PNPS for further assays. Background is defined in text.

To enhance sensitivity and minimize variability, a number of parameters affecting the assays were examined. In the experiments reported here, the PNPS adsorbed to the plates consistently at pH 9.8. Some workers have found success in direct coating of PNPS onto microdilution plates (2, 12, 18), whereas others have failed (1, 5, 18-20). Perhaps the dis-

crepancies in adsorption reported in the literature are a reflection of the type of solid supports used, the coating buffer and the conditions of precoating. In the present study, these questions were not pursued further.

The optimal concentration of PNPS was 3.5  $\mu\text{g}$  of Pneumovax per ml (Fig. 1). In studies reported previously, a range (1 to 200  $\mu\text{g/ml}$ ) of concentrations of PNPS have been used for detecting anti-PNPS antibodies (1, 2, 5, 12, 17-19).

Liquid-phase absorption with PNPS of paired pre- and postimmunization sera caused a decrease in IgG antibody levels (Fig. 2). The reason for the inability to absorb out all of the antibody is a reflection of the absorption technique. Liquid-phase absorption is inefficient in removing all of the antibody in the serum samples, as competition between fluid- and solid-phase antigens for the antibody occurs unless high concentrations of fluid-phase antigen are used. Another possibility is that a component in the Pneumovax may be selectively binding to the wells in preference to the other components. In this case, there would be overrepresentation of antigen on the solid phase compared with the liquid phase and relative inability to absorb out antibody.

The EIA was used to measure serum IgG, IgA, and IgM antibody concentrations following immunization of normal adults with Pneumovax. Defining seroconversion as at least two times the intrarun coefficient of variation, all of these controls showed IgG seroconversion when tested after an average postimmunization period of 6 weeks (Table 1). One of the controls (control D in Table 1) failed to have any IgA response, and one (control E) did not have an IgM response.

Measurement of total serum IgG concentrations in pre- and postimmunization sera from a number of immunized individuals did not show any significant change over that period (data not shown). Therefore, the increase in the PNPS antibody concentrations detected by EIA between the pre- and postimmunization sera are not a result of a nonspecific increase in the IgG concentration of the sera.

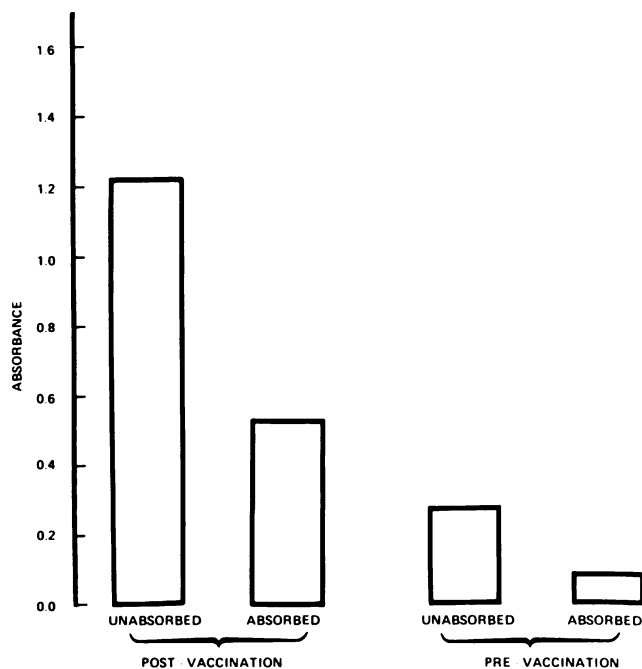


FIG. 2. Liquid-phase absorption followed by detection of IgG antibody of a representative pair of pre- and postimmunization sera. Background absorbance was 0.07.

TABLE 1. Isotype response of individual control subjects to PNPS

Control	Response <sup>a</sup> of the following subject to PNPS:											
	A		B		C		D		E		F	
	Pre <sup>b</sup>	Post <sup>c</sup>	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
IgG	16.1	130.5	153.1	3,840.0	34.2	218.3	4.0	52.4	43.2	128.3	7.4	37.7
IgA	0.8	13.7	46.6	68.7	1.7	4.3	19.1	15.6	2.8	6.7	2.6	42.3
IgM	18.2	247.6	60.1	114.2	121.9	254.5	55.6	381.7	852.2	765.0	144.7	281.8

<sup>a</sup> 10<sup>3</sup> PNPS antibody units (see text).

<sup>b</sup> Pre, Prevaccination.

<sup>c</sup> Post, 4 to 8 weeks postvaccination.

Thus, if seroconversion as defined is used as an index of sensitivity, the assay could be said to be 100% sensitive for IgG and 92% sensitive for IgA and IgM. Unfortunately we have no similar data for specificity, which would have required testing unimmunized controls at weekly intervals for 6 weeks to determine the proportion who failed to seroconvert over that interval.

In initial experiments, a series of paired preimmunization sera and 4- to 8-week postimmunization sera were titrated and the absorbance versus serum dilution curves were compared (results not shown). The shapes of paired pre- and post-sera dilution curves were similar for individual pairs of sera, confirming previous findings (5, 18). This suggests that the antibody measured before and after immunization has equal affinity for the antigen phase in the one individual. Koskela and Leinonen (15) also noticed no marked variation of the slopes of titration curves between serum samples from different patients. However, this was not the observation of the present study, in which titration curve slopes were found to vary between individuals.

Samples taken from 10 hypogammaglobulinemic patients just prior to intravenous immunoglobulin administration were assayed. In these patients, preinfusion IgG concentrations represent small amounts of endogenous IgG production and residual IgG 1 month after the previous infusion of immunoglobulin. The PNPS IgG antibody levels were low or undetectable. This was consistent with the low preinfusion serum IgG concentrations in these patients. They also did not have IgA or IgM PNPS antibodies, consistent with the absence of these immunoglobulins (less than 1 IU/ml) in their serum. Residual IgG PNPS antibody found in the sera of the above patients was likely to have been from the administered immunoglobulin, especially since antibodies to cell wall polysaccharides are to be found in most normal human sera. In the present study, IgG antibody to PNPS was shown to be present in the standard human serum ASPS 78/1 (22) and in normal human gamma globulin. Recently, Hetcherington and Giebink (11) demonstrated opsonic activity to type 14 pneumococcal polysaccharide in intravenous immunoglobulin preparations.

Cord blood sera have IgG antibody to PNPS but no IgM antibody, although the IgG antibody and opsonic activity levels are said to be significantly lower than in maternal serum (6). In the present study, IgG antibody consistent with adult IgG levels was found in all except one of the cord sera. There was no IgM or IgA antibody in any cord serum samples tested.

In the sera of infants aged 6 to 12 months, IgG antibody levels were low. IgA antibody was not detected, and IgM antibody also was low.

Evidence has been presented of the greater ability to discriminate between patient groups using the results of antibody levels to 12 polysaccharide types compared with

using the geometric mean of those results (10). This would seem to imply that discriminatory information would be lost by using the overall responses as we have done. However, no data were given for the results of testing new samples using the calculated discriminant function, which detracts considerably from what could potentially be an important finding. On the other hand, our aim has been to devise a simple technology that could be widely and cheaply applied.

The isotype-specific EIA described in this report uses commercially available pneumococcal antigen which adsorbs readily to the solid phase chosen, and it is capable of demonstrating seroconversion in healthy adult volunteers. This assay may be useful in determining the antibody status of at-risk patients, such as those undergoing splenectomy, and offers a simple means of determining the response to immunization.

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