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Modification of a Direct Enzyme-Linked Immunosorbent Assay for the Detection of Immunoglobulin G and M Antibodies to Pneumococcal Capsular Polysaccharide

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In contrast to the usual indirect enzyme-linked immunosorbent assay (ELISA) method for detection of antibody responses, a modified direct ELISA technique was used to measure immunoglobulin G (IgG) and IgM responses to pneumococcal capsular types 1, 3, 9N, and 23F in humans. Individual capsular polysaccharides were covalently bound to poly-L-lysine before adsorption to the solid phase. The coupling reaction was enhanced by maintenance of a constant pH of 8.2 after the addition of all reactants. The evaluation of four diluents (phosphate-buffered saline [PBS]–Tween; PBS-Tween plus 10% fetal calf serum; PBS-Tween plus 10% bovine serum albumin; and PBS-Tween plus 20% normal goat serum) showed that the sensitivity and specificity of the assay was increased with normal goat serum (10-fold). Serum samples from 10 subjects immunized with polyvalent pneumococcal vaccine were tested by direct ELISA and by radioimmunoassay. At 4 weeks postimmunization, the ELISA method showed that IgG was the predominant antibody and that IgM responses were lower or had diminished. Isotype shifts during this period would have been undetected by the radioimmunoassay method. The changes in antibody response measured by ELISA were comparable to the radioimmunoassay results. The direct ELISA method for the detection of antipneumococcal capsular antibody has been found to be a sensitive and reproducible assay for the detection of IgG and IgM antibodies.

The use of polyvalent pneumococcal capsular vaccines has been shown to decrease significantly the incidence of disease caused by vaccine-type *Streptococcus pneumoniae* (2, 14). The majority of individuals in whom this disease occurs are immunologically compromised (2). Accordingly, the variable antibody response to the pneumococcal capsular vaccine displayed by this patient population has prompted the development of a rapid and reliable assay for the measurement of these antibodies.

A number of methods are available for detecting pneumococcal antibodies. Of these, only the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) offer the required specificity and sensitivity (1, 8, 13). However, because of the poor adsorption of pneumococcal capsular polysaccharide (PPS) antigen to polystyrene cuvettes, many ELISA procedures employ an indirect antibody-bound antigen phase (3, 5, 6, 11). Unfortunately, this is an indirect technique which requires the use of highly purified, type-specific antibodies to decrease nonspecific background activity and increase sensitivity. In addition, these assays require lengthy incubation times.

A method of coupling the PPS to protein has been described by Gray (9). Our modification of the coupling method and the assay has increased the sensitivity and decreased the incubation time. This paper describes a direct ELISA for the detection of immunoglobulin G (IgG) and IgM antibodies to some of the PPS types contained in the 14-valent pneumococcal vaccine (Pneu-Imune; Lederle Laboratories, Pearl River, N.Y.).

MATERIALS AND METHODS

Sera. Serum samples were obtained from healthy adult volunteers who had received a single 0.5-ml subcutaneous injection of 14-valent vaccine (Lederle Laboratories) containing 50 μg of each PPS (types 1, 2, 3, 4, 6A, 8, 9, 12, 14, 18C, 19, 23, and 25 [American nomenclature]). Serum samples were taken immediately before and 2, 3, and 4 weeks after vaccination. Additional vaccine sera also were obtained through the courtesy of Raymond Smith (Veterans Administration Hospital, Albany, N.Y.), David Bentley (Monroe Community Hospital, Rochester, N.Y.), and Hilaire Meuwissen (Albany Medical College, Albany, N.Y.). All serum samples were frozen promptly and stored at −70°C.

Coupling of antigen to poly-L-lysine. PPS types 1, 3, and 23 were obtained from the American Type Culture Collection, Rockville, Md., and type 9 was obtained from Kenneth Amiraian, New York State Department of Health, Albany, N.Y. Each PPS type was coupled to poly-L-lysine (molecular weight ca. 50,000; Sigma Chemical Co., St. Louis, Mo.) as follows. PPS was diluted to 1,000 μg/ml in distilled water and stored at 4°C. A 200-μl portion of PPS was added to 1.0 ml of 0.01 N NaOH and stirred for 10 s. Cyanuric chloride (1.0 mg; Sigma) was then added and mixed vigorously for 10 s. To this solution, 0.2 ml of 0.02% poly-L-lysine solution was added and stirred for 10 s. The pH of this solution was continuously monitored with a pH meter and adjusted to 8.2 with the addition of NaOH. The mixture was incubated for 2 h at 4°C with occasional stirring.

ELISA. The assay method for ELISA was a modified version of that described by Engvall and Pearlmann (8). Each PPS coupled to poly-L-lysine was diluted in 0.1 M Na_2CO_3 buffer (pH 9.8) containing 0.02% NaN_3 and added in 200-μl portions to wells of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.). The plate was incubated overnight at 4°C and washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween). Excess PBS was removed by flicking the plate. Serum specimens were diluted in PBS-Tween, PBS-Tween

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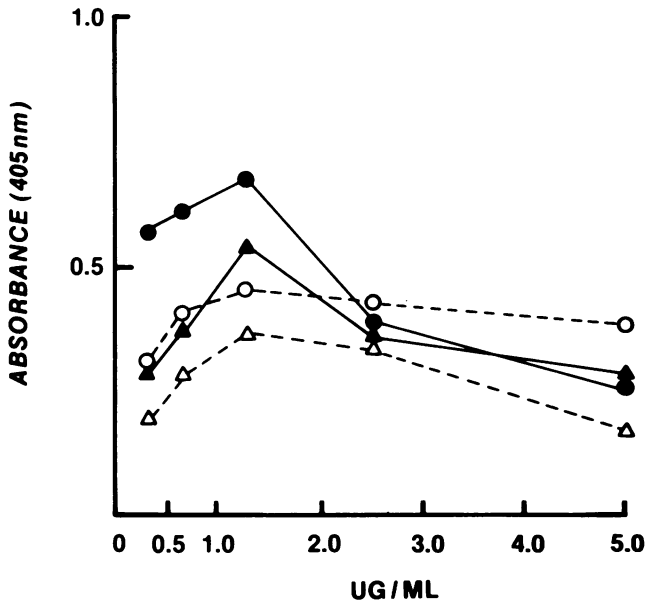

FIG. 1. Optimal antigen concentration for coating plates. PPS types 1 (○), 3 (▲), 9 (●), and 23F (△) were coupled to poly-L-lysine, diluted in coating buffer, and bound to the polystyrene by overnight incubation at 4°C. The plates were washed and reacted with a 1:100 dilution of human serum known to contain anti-PPS antibody (as determined by RIA). These were then reacted with enzyme-labeled anti-human IgG and finally with the enzyme substrate. Optimal coating of the polystyrene occurred at a PPS concentration of 1.25 μg/ml for all four PPS types tested.

containing 10% bovine serum albumin, PBS-Tween containing 10% fetal calf serum, or PBS-Tween containing 20% normal goat serum. Each serum dilution was tested in quadruplicate for each run. The plate was incubated for 2 h at 37°C in a moist chamber and washed three times with PBS-Tween. Alkaline phosphatase-conjugated goat anti-human IgG (γ-chain specific) or IgM (μ-chain specific) (Sigma) diluted 1:1,000 in one of the above buffers was added to each well in 100-μl amounts. After an incubation period of 2 h at 37°C, the plate was washed three times with PBS-Tween. The substrate, *P*-nitrophenyl phosphate (Sigma), was diluted to 1 mg/ml in 10% diethanolamine buffer (pH 9.8) containing 1 mM $MgCl_2$ and 0.02% NaN_3 and was added in 200-μl amounts to each well. The plate was incubated at 37°C, and the change in optical density was determined at various times with an eight-channel photometer (Titertek Multiskan; Flow Laboratories, Inc., McLean, Va.) at 405 nm.

The nonspecific absorbance of reagents to the plastic of the microtiter plate was determined in parallel controls. The antibody control well contained all the reagents (i.e., sample serum, conjugate, and substrate) except for the poly-L-lysine-bound PPS, and the antigen control contained all reagents except the sample serum. The ability of poly-L-lysine to react nonspecifically with the test sera was also tested by performing the conjugation step in the absence of the PPS antigens.

RIA. RIA results were obtained through the courtesy of Gerald Schiffman, Downstate Medical Center, Brooklyn, N.Y.

RESULTS

In the procedure used to couple the PPS to poly-L-lysine, the pH of the initial solution was between 11.9 and 12.5. After the addition of cyanuric chloride, the pH of this solution ranged from 9.6 to 2.3, and at the end of the 2-h incubation period the pH was always <2.5. Because of this variability in pH throughout the reaction, the poly-L-lysine was added only after the pH had dropped to 8.2, and the pH was then maintained at 8.2 by the addition of 1 N NaOH. After 15 min, the decrease in pH stopped and further adjustment was not necessary for the 2-h reaction period at 4°C. This procedure produced constant results with all the antigens used.

To determine the appropriate concentration of the reagents, various concentrations of antigen were added to the wells, followed by various serum dilutions. The optimum antigen concentration for the four PPS types tested was 1.25 μg/ml (Fig. 1). A decrease in absorbance occurred at higher antigen concentrations. The optimum serum dilution for a 10-min reaction time was 1:100. The reaction time consisted of the period of incubation of the substrate with the enzyme-labeled goat anti-human immunoglobulin, and this time increased (up to 2 h) as the antibody concentration decreased. The maximum dilution of the immune serum which gave a measurable change in optical density was 10^{-5}; however, this increased the reaction time to 2 h. No detectable reaction occurred when poly-L-lysine was used in the assay without the PPS antigens.

The sensitivity of the assay was also influenced by the diluent used. The dilution of both the serum and the conjugate in PBS-Tween, PBS-Tween with 10% fetal calf serum, and PBS-Tween with 10% bovine serum albumin resulted in an increase in nonspecific absorption (Fig. 2). This background activity was minimized when PBS-Tween with 20% normal goat serum was used as the diluent; this increased the sensitivity of the assay so that a 10^{-5} dilution of serum gave a reproducible result.

Standard curve construction. The linearity of the ELISA assay results was tested by diluting a standard serum whose antibody concentration was determined as 5,496 ng of AbN per ml by RIA and by plotting the log of the dilution against the optical density (absorbance) at 405 nm (Fig. 3). The curve was linear over a 10-fold dilution range, corresponding to antibody concentrations of 0.5 to 5.0 ng of AbN per ml.

Precision of ELISA. The assay variance with quadruplicate samples per assay was excellent; each test serum varied by <0.05 absorbance units. To determine the reproducibility, one pair of pre- and postvaccination sera was tested on 9 separate days with fresh preparations of PPS type 9 antigen conjugated to poly-L-lysine. The values (absorption units) obtained in separate determinations ranged from 0.024 to 0.202 and from 0.516 to 1.150 for pre- and postvaccination sera, respectively (Table 1). Calculation of the net absorption units for this sample also showed a large variation in reproducibility, with all the values falling in a range between 0.472 and 1.072. This clearly demonstrated the need to run paired sera with the same antigen conjugate preparation or with a reference serum.

Detection of antibody class. Both the IgG and IgM response of pre- and postvaccination sera were evaluated with PPS types 1, 3, 9, and 23. A vigorous antibody response as measured by ELISA occurred with PPS types 3 and 9 (Table 2). Very little change between pre- and postvaccination sera was seen with type 1, and there was a relatively low IgG response and no IgM response with type 23F. The results were similar for 10- and 30-min incubation times.

The RIA results of 10 sera were directly compared with the responses obtained for IgG and IgM by the direct ELISA method (Table 3). Of these sera, nine showed an increase in

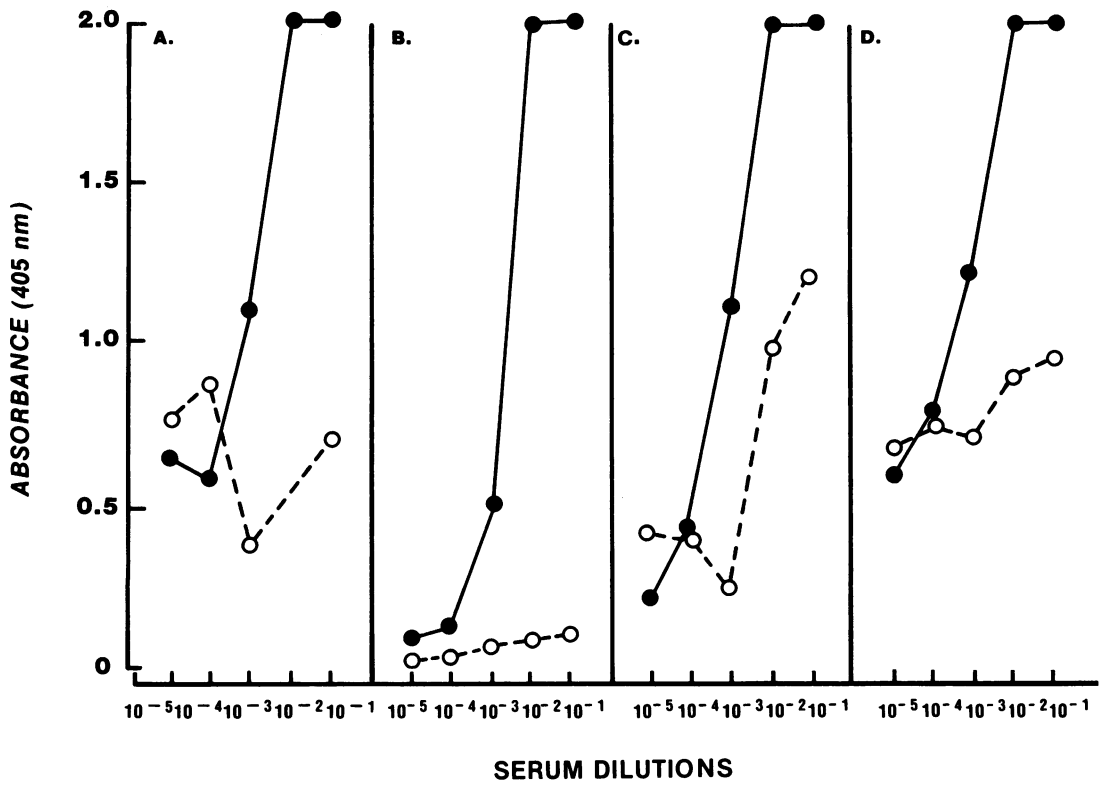

FIG. 2. Effects of diluent on ELISA sensitivity. Wells were coated with 1.25 μg of PPS–poly-L-lysine per ml, and various dilutions of a standard serum (prevaccination [○] and postvaccination [●]) were made in PBS-Tween alone (A) or PBS-Tween supplemented with 20% normal goat serum (B), 10% fetal bovine serum (C), or 10% bovine serum albumin (D). The plates were washed, reacted with enzyme-labeled anti-human IgG for 2 h at 37°C, and developed by incubation with the enzyme substrate for 30 min at 37°C. The addition of 20% normal goat serum resulted in increased sensitivity at all serum dilutions tested.

absorbance corresponding to the change observed by RIA. Two discrepancies were observed in that the measured ELISA response was high compared with the RIA response. These occurred in the IgG response of patient 7 to type 9 PPS and of patient 6 to type 3 PPS. Although the magnitude of the ELISA response was greater in relation to the RIA values, the direction of the response was the same with virtually no change for patient 7 and with a moderate increase in IgG and a large increase in IgM response for patient 6. The RIA employed measured total antibody concentration; thus, these discrepancies may be due to the measurement of other immunoglobulins, such as IgA, by RIA which were not measured in our study. This may also explain the greater magnitude of change which can be seen by RIA between pre- and postvaccination sera which is not detectable by ELISA in some patient samples.

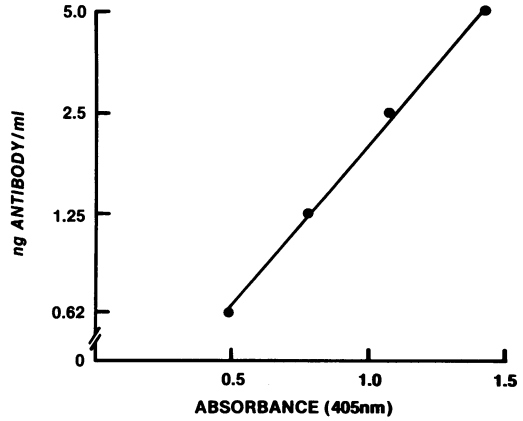

FIG. 3. Standard curve construction for PPS type 9 by the ELISA method. The curve was constructed by plotting the log of serial dilution of a standard serum against the optical density (absorbance) at 405 nm. The curve is linear over a 10-fold dilution range.

TABLE 1. Interrun reproducibility: IgG response of control serum to PPS type 9

Run no.	IgG response (absorption units)		
	Postvaccination serum	Prevaccination serum	Corrected[a]
1	0.880	0.069	0.811
2	1.113	0.202	0.911
3	0.516	0.040	0.476
4	0.948	0.024	0.924
5	0.823	0.037	0.786
6	0.852	0.104	0.748
7	1.150	0.078	1.072
8	0.606	0.024	0.582
9	0.852	0.035	0.817

[a] Within-run postvaccination serum value minus prevaccination serum value.

TABLE 2. Effect of incubation time and response of pre- and postvaccination sera in the direct ELISA[a]

Incubation time (min)	PPS type	Response (absorption units)			
		IgG		IgM	
		Pre	Post	Pre	Post
10	1	0.004	0.007	0.009	0.012
	3	0.038	0.183	0.029	0.379
	9	0.130	1.013	0.035	0.405
	23 F	0.028	0.063	0.003	0.015
30	1	0.010	0.015	0.025	0.031
	3	0.073	0.538	0.066	1.197
	9	0.401	>2.000	0.100	1.050
	23 F	0.086	0.221	0.013	0.045

[a] The patient was vaccinated with the 14-valent pneumococcal vaccine, and pre- and postvaccine sera (Pre and Post, respectively) were obtained 4 weeks apart.

DISCUSSION

The direct ELISA method described in this paper is a sensitive and specific assay for the determination of antibody responses in individuals immunized with 14-valent pneumococcal vaccine. The method shows excellent intra-run precision, with all values for multiple-run specimens differing by less than 0.05 absorption units. This represents in part the uniformity of the PPS preparation used to coat the microtiter plates. Two modifications which contributed to this precision are the preparation of the PPS–poly-L-lysine conjugate at a constant pH of 8.2 and the addition of normal goat serum to the PBS-Tween used as a diluent. The specificity of the PPS–poly-L-lysine complex for the type-specific antipneumococcal antibody can be seen from the difference in optical density observed between pre- and postimmunization sera. This specificity was also demonstrated by the lack of color development in wells coated with poly-L-lysine only. The direct ELISA also circumvents the indirect or sandwich-type assay which requires highly purified and specific antibody to PPS. The elimination of this complication has resulted in a method that is relatively simple and rapid in comparison to the indirect assays described previously (3, 11).

A problem commonly encountered with ELISA procedures is the batch-to-batch variability in the amount of antigen adsorbed to the solid phase. An attempt to standardize the conjugation of the antigen to poly-L-lysine by maintaining a constant pH of 8.2 resulted in a decrease in variability; however, the variability which remained was such that a control or standard serum must be run with each new batch of coupled antigen so that sera tested with different lots can be compared. This standardization has been successful with other types of direct ELISA (11).

The sensitivity of the assay was found to be equivalent to 0.5 ng of AbN per ml as determined by RIA. The sensitivity of the assay was also found to be affected by the diluent used. Optimal results were obtained when the sample serum

TABLE 3. Comparison of ELISA and RIA results with PPS antigen types 1, 3, 9, and 23F for 10 patients

Patient no.	Serum	PPS type 1			PPS type 3			PPS type 9			PPS type 23F		
		RIA[b]	ELISA[a]		RIA	ELISA		RIA	ELISA		RIA	ELISA	
			IgG	IgM		IgG	IgM		IgG	IgM		IgG	IgM
1	Pre	531.0	0.103	0.026	0.0	0.035	0.024	265.5	0.020	0.023	310.6	0.050	0.027
	Post	693.0	0.110	0.018	357.9	0.299	0.032	1,085.2	0.703	0.043	1,383.3	0.268	0.014
2	Pre	2,192.8	0.516	0.000	247.8	0.132	0.022	283.7	0.317	0.000	1,730.9	0.340	0.000
	Post	2,331.2	0.517	0.072	5,800.6	>2.000	1.003	1,167.7	0.783	0.054	10,159.2	1.241	0.000
3	Pre	548.7	0.076	0.050	173.5	0.038	0.064	136.7	0.033	0.015	142.4	0.065	0.012
	Post	2,324.8	0.269	0.265	3,569.7	1.004	1.360	1,913.1	>2.000	0.493	1,519.7	0.170	0.014
4	Pre	424.2	0.046	0.118	4.5	0.020	0.073	41.4	0.146	0.081	593.7	0.000	0.000
	Post	790.7	0.068	0.091	766.3	0.133	0.273	487.3	0.667	0.202	3,668.9	0.111	0.089
5	Pre	2,071.7	1.180	0.404	1,987.5	0.260	0.381	999.1	>2.000	0.440	2,641.0	>2.000	0.050
	Post	1,349.5	0.698	0.220	1,271.9	0.133	0.218	1,006.9	>2.000	0.361	2,096.0	>2.000	0.030
6	Pre	2,316.6	>2.000	0.420	0.0	0.288	0.159	236.8	>2.000	0.716	1,391.8	>2.000	0.091
	Post	2,766.1	>2.000	0.609	1,771.5	0.347	0.460	664.7	>2.000	0.917	11,465.6	>2.000	0.115
7	Pre	996.2	1.366	0.017	0.0	0.079	0.009	11.9	0.924	0.000	363.4	0.683	0.000
	Post	711.2	1.358	0.021	0.0	0.093	0.003	18.9	1.011	0.000	343.1	0.640	0.006
8	Pre	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Post	2.4	0.030	0.019	0.0	0.019	0.043	1.5	0.000	0.000	119.8	0.005	0.035
9	Pre	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Post	718.0	2.000	0.312	11.7	0.268	0.076	3,161.0	>2.000	0.081	8,206.0	1.540	0.056
10	Pre	NT	NT	NT	NT	NT	NT	200.0	1.192	0.021	NT	NT	NT
	Post	NT	NT	NT	NT	NT	NT	5,496.0	>2.000	0.384	NT	NT	NT

[a] A fixed serum dilution (1:100) was used in all assays for both pre- and postvaccination samples (Pre and Post respectively). ELISA values are expressed as absorption units measured at 405 nm of antigen-coated cuvettes (wells) minus background absorbance in antigen-free cuvettes (wells). NA, Not available; NT, not tested.

[b] Antibody levels by RIA are expressed in nanograms of AbN per milliliter.

and the developing antiserum were diluted in PBS-Tween buffer containing 20% normal goat serum. The serum proteins in this buffer probably adsorbed to the solid phase at sites not occupied by the PPS–poly-L-lysine complex, resulting in a decrease in the amount of human proteins which were nonspecifically adsorbed to the polystyrene. Of a number of serum proteins tested from various species, the normal goat serum gave the best results, presumably because the developing antibody conjugate was raised in a goat, thus also eliminating nonspecific interspecies reactivity.

The qualitative responses of the sera from the vaccinees determined with the direct ELISA method agreed with those determined with the standard RIA in that the responses were similar and changed in the same direction. A problem arises in interpreting the magnitude of the change for quantitating the antibody response and in correlating that response to the establishment of immunity. A number of investigators have described various ways of converting ELISA units into antibody levels (5, 7, 11). Of these, the method described by Eisenstein et al. (7) seems to be the most generally applicable. The antibody concentration of a standard serum is determined by a precipitation test; this serum is then included in every assay. A standard curve is established, and the antibody concentration of unknown sera can be extrapolated from the linear portion of the curve. We also obtained a linear response over a 10-fold range of antibody concentrations. It is possible that, given a sufficiently high-titered serum, this type of relationship can be established and the antibody response can be quantitated.

An advantage of the ELISA is that by using the appropriate developing serum, changes in isotype in response to the vaccine or infection can be detected. In the present study, IgG and IgM response to the polyvalent vaccine was determined, and it was found that with the four PPS types tested the IgG response at 4 weeks postvaccination was greater than the IgM response. The changes detected by ELISA corresponded well with those detected by RIA in that the shifts were in the same direction by both methods. It has been difficult to correlate ELISA measurements of antibody concentration with the results of the RIA method. The difficulty has been attributed to the fact that RIA measures total antibody of both high and low avidity, whereas ELISA methods measure only one isotype and only high-avidity antibodies (4). Although it has been suggested that an antibody level of 300 ng of AbN per ml as measured by RIA is protective, a clinical correlation of antibody class with concentration in terms of protection has not been established for either method (10, 12, 13). It would be of interest to study this problem further and to establish a correlation between antibody concentration and isotype measured by ELISA and protection against pneumococcal infection. An evaluation of this type could render the direct ELISA assay described in this paper a potent diagnostic tool for the management of patients at risk for pneumococcal infections.

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