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Enzyme-Linked Immunosorbent Assay for Measurement of Antibodies Against Pneumococcal Polysaccharide Antigens: Comparison with Radioimmunoassay

LYNN T. CALLAHAN III, ALLEN F. WOODHOUR, JEFF B. MEEKER, AND MAURICE R. HILLEMAN*

Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

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An enzyme-linked immunosorbent assay (ELISA) for measuring antibodies against each of 14 polysaccharides in contemporary pneumococcal vaccine is described, and the findings of tests of paired sera from vaccinated human subjects are compared with those obtained by radioimmunoassay. The findings were in very poor agreement, and this appears to be due to the lesser ability of the ELISA procedure to measure antibody of low avidity. The ELISA procedure described here is not considered to be a satisfactory substitute for radioimmunoassay for measuring antibody responses to pneumococcal vaccine.

Clinical studies attending the evaluation of polyvalent pneumococcal polysaccharide vaccines (9) require a specific quantitative assay for homologous antibody responses in the sera of human subjects who receive the vaccine. Present measurement of humoral antibody is by radioimmunoassay (RIA) (G. Schiffman and R. Austrian, Fed. Proc. **30**:658, 1971). This assay, though technically reliable, is prohibitively expensive, and a more practical procedure is needed. Simplification was sought by employing the enzyme-linked immunosorbent assay (ELISA) for antibody measurement. The present report describes an ELISA procedure that was developed here and used in comparative testing of sera from vaccinated human subjects by both the RIA and ELISA procedures. Although an ELISA procedure for the detection of antibodies to pneumococcal polysaccharides has been reported (1), the one described here differs primarily in being a semiautomated assay for the measurement of the total complement of immunoglobulin G, M, and A antibodies and incorporates an immunoglobulin "bridge" between the test antibody and enzyme conjugate for purpose of amplifying the reactions.

MATERIALS AND METHODS

Human sera. Adult human subjects, who had given voluntary informed consent, each received a single 0.5-ml injection of 14-valent pneumococcal vaccine (9) containing 50 μg each of types 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F, and 25 polysaccharide antigen. Blood samples were collected immediately before and 4 weeks after vaccination, and the sera were stored frozen until assayed.

RIA. The RIA procedure was as described by Schiffman and Austrian (Fed. Proc. **30**:658, 1971) and was an adaptation of the RIA method of Farr (4). The radioactive type-specific pneumococcal polysaccharides used in the tests were obtained from Gerald Schiffman. Serum titers in the assay were expressed as nanograms of antibody nitrogen per milliliter of serum, and a twofold or larger difference in antibody titer between pre- and postvaccination serum samples, in the same test run, was considered to be significant.

ELISA reagents. (i) Pneumococcal polysaccharides. The 14 lyophilized polysaccharides were prepared by Thomas H. Stoudt and his associates, Merck Sharp & Dohme Research Laboratories, Rahway, N.J. Considerable differences were observed among different preparations of the same serotype polysaccharide in coating of the polystyrene wells in which the tests were carried out. Selection of acceptable antigens was made and only a single preparation of each serotype antigen was used in the tests.

(ii) Anti-human immunoglobulin prepared in goats. The human globulin fraction used to immunize the goats was purified from a sample of adult normal human serum by ammonium sulfate fractionation (50% saturation) and gel permeation chromatography on a Sephacryl S-200 (superfine; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column. The purified globulin fraction contained 1.2 mg of protein per ml as measured by the Lowry method (5) and was free from detectable albumin as judged by counter immunoelectrophoresis, using rabbit anti-human albumin antibody as the indicator. The human globulin fraction was emulsified with an equal volume of Freund incomplete adjuvant and was given to three newly weaned goats, 1 to 2 months of age, that had been selected by direct ELISA, using rabbit anti-goat immunoglobulin conjugate (described below), for the absence of antibody against any of the 14 pneumococcal polysaccharides included in the vaccine. The globulin fraction

was given subcutaneously in a 2.5-ml volume into each leg (5.0 ml total) on three occasions at weekly intervals. Bleeding was 2 weeks after the third dose. None of the goat antisera contained detectable antipneumococcal antibody as tested for by the ELISA procedure. Furthermore, antibodies against human immunoglobulins G, M, and A were present in high titers.

(iii) Anti-goat immunoglobulin prepared in rabbits and conjugated with alkaline phosphatase. The immunoglobulin G fraction of rabbit anti-goat globulin (Cappel Labs) was conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.; catalog no. P-4502, type VII, 1140 U/mg of protein) by a modification of the method of Engvall and Perlmann (3). The conjugate did not react with any of the 14 pneumococcal polysaccharides by the ELISA procedure.

ELISA procedure. The ELISA tests were carried out in Microelisa Substrate Plates (Dynatech Laboratories, Alexandria, Va.). Individual reconstituted polysaccharides diluted in 0.01 M phosphate-buffered saline (pH 7.4) to a 10.0-μg/ml concentration were used in 250-μl amounts to coat appropriate wells (The 10-μg/ml concentration was used because it appeared to be optimal, based on titrations with the 14 pneumococcal polysaccharides.) After overnight incubation at 37°C, the wells were washed four times with phosphate-buffered saline, containing 0.05% Tween 20 (Fisher Scientific Co., Pittsburgh, Pa.), by aspiration in a programmed sequence, using an automatic microtiter plate washer (Dynawasher, Dynatech). This washing procedure was consistent throughout. Bovine serum albumin (1%, 250-μl amounts) in phosphate-buffered saline then was added to each well; lids were placed on all plates immediately before storage at 4°C for 3 days. The plates then were washed, inverted with lids in place, and further stored at 4°C until used. In the assays of sera to be tested, the prevaccination sera diluted 1:100 and 1:1,000 and the postvaccination sera diluted 1:100, 1:1,000, and 1:10,000 with phosphate-buffered saline containing 0.05% Tween 20 and 0.05% bovine serum albumin were added in 250-μl amounts to duplicate wells that had been coated with one of the pneumococcal polysaccharides. The plates were incubated at 37°C for 1 h, after which time they were washed and 250-μl amounts of goat anti-human immunoglobulin diluted 1:1,000 in phosphate-buffered saline–Tween 20–bovine serum albumin solution were added. After further incubation for 1 h at 37°C, the plates were washed again, and 250-μl amounts of rabbit anti-goat immunoglobulin conjugate diluted 1:2,000 in phosphate-buffered saline containing 10% bovine serum albumin were added to each well. This was followed by an additional incubation for 1 h at 37°C. Finally, phosphatase substrate in 0.54% Na_2CO_3–0.02% $MgCl_2 \cdot 6H_2O$ (pH 9.8; Sigma catalog no. 104) at a concentration of 1 mg/ml was added to each well in 250-μl amounts. Incubation was carried out for 3.5 h at 37°C in tests with types 2, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, and 19F and for 18 h with types 1, 3, 23F, and 25. The reactions were quenched by the addition of 25 μl of 5 N NaOH, and the optical density was read for each well at 400 nm in a Stasar II spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a data list attachment. For purpose of constucting the standard curves, high-titer positive serum samples were assayed in duplicate in each test for each pneumococcal serotype by a procedure identical to that described above. The standard sera were taken from persons who had received the pneumococcal vaccine and were selected for titers in the range of 2,000 to 22,000 ng of antibody nitrogen per ml as measured by RIA. Negative control sera with <200 ng of antibody nitrogen per ml were included in each test. Serial twofold dilutions of standard sera were tested in the range of 1:2,000 to 1:64,000 dilution for type 6A antigen and in the range of 1:200 to 1:6,400 for all other types. For control purposes, each human serum sample was assayed at the lowest dilution in wells that were not coated with antigen; nonspecific adsorption of antibody to the plastic did not occur. A stylized version of the layering of reagents in the ELISA procedure is given in Fig. 1.

Data analysis. Standard curves were constructed for each of the two standard sera that were purposely tested on two separate plates. Analysis of covariance of the log optical density against the log serum dilution was used to compare the replicate curves, including tests for curvature and parallelism. The standard curves were linear and parallel, and curvature, when present, was found only at the high and low extremes of the serum dilutions.

The principal finding in this investigation was the measure of fold increases in antibody titer in patient serum samples taken before and after vaccination, and comparison was made of the fold increases in titer as measured by ELISA and RIA. Direct comparisons of the titers obtained in the two assays were rejected, since plotting of the pre- and postvaccination serum samples together, giving 16 to 20 observations per serotype, produced a two-clump scattergram with a false high correlation, and the members of the same pair were not independent since the pre- and postvaccination sera were from the same individuals. Pearson correlation (7) was used to compare the fold increases. Spearman (rank) correlation (7) also was used since it is less susceptible to bias that might appear as a result of truncating RIA titers at the higher-titer values. Agreement in the numbers of twofold and fourfold increase in antibody was assessed by the McNemar test (6).

RESULTS

Construction of standard curves by ELISA. Standard serum samples for each of the 14 antigen types were assayed by ELISA and compared with RIA-negative serum samples. A single dilution of 1:200 of the negative sample was used for the comparison. Antigen control wells (not coated with antigen but containing serum at a 1:200 dilution) were included as a test for nonspecific adsorption. Figure 2 presents the results of testing a standard and negative serum sample with type 9 pneumococcal polysaccharide and exemplifies what was observed for the other pneumococcal types.

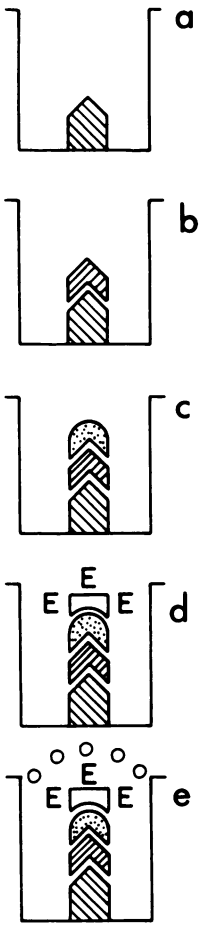

FIG. 1. *Schematic diagram of ELISA for detection of human antibodies to pneumococcal polysaccharides (modified from Voller et al. [8]). (a) Polysaccharide adsorbed to well. (b) Human serum (anti-polysaccharide) added to well. (c) Goat anti-human immunoglobulin added. (d) alkaline phosphatase-linked rabbit anti-goat immunoglobulin added. (e) Substrate solution added. If there is any alkaline phosphatase attached to the solid phase, it converts the colorless substrate to a yellow solution, which can be detected colorimetrically at a wavelength of 400 nm.*

Comparison of findings obtained by the ELISA and RIA procedures. It is important to compare the fold increases in amount of antibody against each serotype measured by the RIA and ELISA test methods. Fold increases in antibody titer of the paired sera in the ELISA procedure were determined for each serotype by comparing the optical density of a given dilution of test serum with the constructed standard curve.

The findings in the comparative titrations of 10 pairs of sera from vaccinated persons are given in Table 1. Only those assays in which RIA showed a twofold or greater increase in titer were included. There was poor correlation in the fold increases in antibody titers measured by the two procedures. They were usually far greater when the sera were assayed by RIA than by ELISA. Good correspondence in values was obtained only for type 1 and 8 polysaccharides, in which the (Pearson and Spearman rank) correlation coefficients were 0.93 and 0.87, respectively, for type 1 and 0.88 and 0.92, respectively, for type 8.

DISCUSSION

Sera from most human beings contain antibody against pneumococcal polysaccharide antigens, as measured by RIA (9). At least 80% of human subjects 2 years of age or older display a twofold or greater increase in homologous antibody against all serotypes when given the polyvalent pneumococcal vaccine. Usually, the responses to vaccination are much greater than twofold. There is no precise information as to what kind(s) of antibody is responsible for immunity against pneumococcal infection, nor is there any defined minimal serum titer value

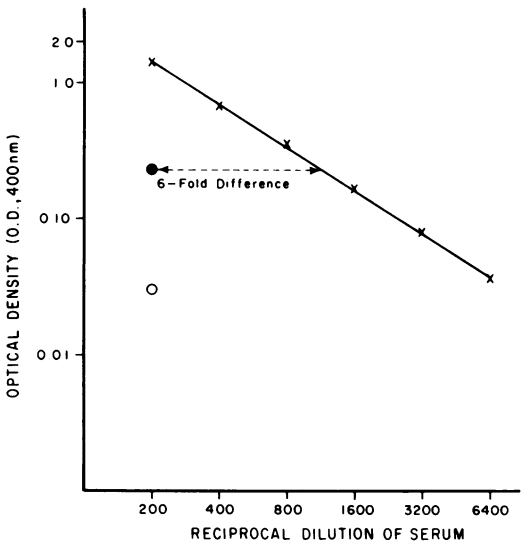

FIG. 2. *Standard curve construction for type 9 pneumococcal polysaccharide by the ELISA method. Mean optical density values of various dilutions of standard serum (×) were used to construct the standard curve, which then was compared with the antigen control (○) and the negative serum control (●). A sixfold difference between the standard and negative serum samples was found. This was typical of findings with the 13 other polysaccharides.*

TABLE 1. *Summary of fold increases in specific antibody in sera from vaccinated persons as measured by ELISA and RIA*

Pneumococcal serotype	No. of serum pairs	No. of persons showing antibody increase				Correlation coefficient (r) and significance (p)			
		≥2×		>4×		Pearson		Spearman rank	
		ELISA	RIA	ELISA	RIA	r	p	r_s	p
1	9	5	6	2	3	0.93	0.0002	0.87	0.0025
2	10	2	7	1	2	−0.23		−0.02	
3	9	0	3	0	1	0.45	0.22	0.61	0.08
4	10	1	9	0	2	0.12	0.97	0.13	0.73
6A	8	5	8	1	8	−0.12		0.19	0.65
7F	10	3	9	1	7	0.31	0.38	0.27	0.45
8	10	3	6	0	4	0.88	0.0007	0.92	0.0002
9N	10	7	8	2	7	0.45	0.19	0.53	0.11
12F	8	2	7	0	4	0.10	0.81	0.07	0.87
14	9	4	7	0	6	0.36	0.34	0.27	0.49
18C	8	5	8	3	7	−0.07		−0.10	
19F	8	2	4	0	4	0.40	0.25	0.20	0.58
23F	9	2	8	0	5	−0.03		0.08	0.85
25	10	0	10	0	9	0.09	0.80	0.40	0.26

needed to establish immunity. In spite of this, current judgments relative to the effectiveness of the vaccine are based on determinations of the fold increase in amount of type-specific antibody, measured by RIA, in the sera of persons taken before and after vaccination.

The RIA procedure is expensive, and there is a limitation in the availability of radiolabeled type-specific polysaccharides needed to conduct the test. The ELISA procedure, which does not require radiolabeled antigen and which can be fully automated, presents obvious potential advantages if the findings in the assay are comparable to those of RIA, for which all judgments for vaccine efficacy are based.

This report describes the development of an ELISA procedure that measures antibodies to pneumococcal polysaccharides and presents the findings of a comparative evaluation of results generated by this procedure and RIA, using sera taken from human subjects who were given pneumococcal polysaccharide vaccine. The findings showed very poor correlation for most serotypes and indicated that the ELISA procedure described here could not be substituted for RIA, at least in its present state of development.

Although unproved, it appeared that the differences in the two test results were related to the variable affinity of antibodies to antigen. RIA measures total precipitable antibody. ELISA measures antibody that still clings to homologous antigen after a series of washing steps carried out during formation of the multilayer complex required to carry the alkaline phosphatase. It appears that much of the antibody is lost if it is of low affinity. Butler et al. (2) recently presented evidence in support of this conclusion in studies in which anti-2,4-dinitrophenyl antibodies were measured in sera by the ELISA procedure and by an RIA procedure similar to that used here. It was clearly shown that increases in affinity of antibody were correlated positively with values obtained by the ELISA method but negatively correlated with values as measured by RIA. ELISA appears to offer no promise at this time as a substitute for RIA. A more meaningful ELISA procedure might be developed once the class(es) of antibody responsible for immunity against pneumococcal infections is known.

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