

Enzyme-Linked Immunosorbent Assay for Determination of Human Antibodies to Group C Meningococcal Polysaccharide (41299)

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Abstract. To study immunity to meningococcal diseases, investigators need a sensitive, serological technique for measurement of the protective antibodies. Although the well-accepted assay for this purpose is the radioimmunoassay (RIA), it requires expensive equipment and uses potentially hazardous reagents which usually have a short shelf life. The enzyme-linked immunosorbent assay (ELISA) has been reported to be as sensitive and reliable a technique as the RIA. We have developed the ELISA for determination of antibodies to group C meningococcal polysaccharide. A successful method for the indirect coating of polystyrene surfaces with polysaccharide antigen, the biochemical purification of the different reagents used and the various optimal ELISA parameters are described. The results of 90 different human sera tested by the ELISA technique were comparable with RIA results of the same sera, and a significant correlation was obtained ($r = 0.86$; $P < 0.001$).

Meningococcal disease is a major cause of mortality and morbidity among children throughout the world and also among many adults in sporadic epidemics (1). Cerebrospinal meningitis outbreaks have continued to occur in Africa, but at the same time the morbidity due to the disease has risen to epidemic proportions in some countries of South America, the Middle East, Southern Africa, Europe, and Asia (2). To study immunity to meningococcal diseases, investigators need a simple and sensitive serological technique for measurement of the protective antibodies. The enzyme-linked immunosorbent assay (ELISA) has been used to detect the immune response to a wide variety of infectious agents (3-5). The ELISA has been shown to be as sensitive as the radioimmunoassay (RIA) (6-8) and can be adapted to large-scale testing. The assay uses an enzyme as the immunoglobulin marker instead of radioisotopes. The ELISA reagents are stable and non-hazardous, and the assay does not require expensive radioactivity detection equipment.

In the present study, we have adapted the ELISA for the measurement of antibodies to group C meningococcal polysaccharide in humans.

Materials and Methods. *Group-specific meningococcal polysaccharide antigen.* Group C meningococcal polysaccharide was isolated from strain C₁₁, group C, *Neisseria meningitidis* (obtained from Dr. J. B. Robbins, U.S. Food and Drug Administration, Bethesda, Md.) according to the method of Gotschlich *et al.* (9).

Horse anti-group C meningococcal antibodies. Horse anti-group C antiserum was prepared according to the method of Alexander *et al.* (10). A partially purified IgG fraction (PPIgG) of horse anti-group C meningococcal polysaccharide was prepared by fractionating the horse antiserum on Sephadex G-200 (Pharmacia, Uppsala) (11). The PPIgG fraction was further purified on DEAE-Sephadex A-50 (Pharmacia, Uppsala) according to the method of The and Feltkamp (12), except that the ammonium sulfate fractionation step was eliminated.

Conjugate. Whole rabbit anti-human IgG serum (Kallestad, Minneapolis, Minn.) was fractionated by three successive precipitations using 40% saturated (NH₄)₂SO₄ followed by purification on DEAE-Sephadex A-50, as for PPIgG.

The purified IgG fraction of rabbit anti-

human IgG was coupled to alkaline phosphatase (Sigma, Type VII) by the one-step glutaraldehyde technique described by Engvall and Perlmann (13). The conjugated enzyme was precipitated with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dissolved in 0.5 ml of 0.08 M Tris-HCl buffer at pH 8 and fractionated by gel filtration using Sephadex G-200 to separate the excess unbound enzyme. The optical density (OD) was monitored at 280 nm by a recording spectrophotometer. The first peak was pooled and 0.1 M MgCl_2 was added to obtain a final concentration of 0.001 M. NaN_3 was added for a final concentration of 0.02%. The conjugate was stored at -20° .

Test performance. The ELISA sandwich technique was a modification of the method of Engvall and Perlmann (13). A 1-ml IgG fraction of horse anti-group C meningococcal polysaccharide (10 μg) in 0.1 M sodium carbonate buffer, pH 9.6, containing 0.02% NaN_3 was added to each polystyrene tube (12 \times 75 mm; Falcon Plastics, Division of Becton, Dickinson, & Co., Oxnard, Calif.). The tubes were incubated in a water bath at 37°C for 3 hr and stored at 4° overnight. The precoated tubes were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween). Then 1 ml of group C meningococcal polysaccharide antigen (5 μg) diluted in PBS-Tween was added to the immunoglobulin-coated tubes. The tubes were incubated in a water bath at 37° for 1 hr. The coated tubes containing the polysaccharide antigen were used immediately in the assay.

The tubes were washed three times with PBS-Tween. One milliliter of serum was added to each tube and the tubes were incubated for 1 hr in a 37° water bath. After washing three times with PBS-Tween, 1 ml of conjugate diluted in PBS-Tween containing 4% bovine serum albumin (BSA) (Sigma, fraction V) was added. The tubes were incubated at 37° overnight (about 16 hr), washed three times with PBS-Tween, and 1 ml of substrate (1 mg *p*-nitrophenyl phosphate/ml 0.05 M sodium carbonate buffer, pH 9.8, containing 0.001 M MgCl_2) was added to each tube. The tubes were incubated in a 37° water bath for 2 hr. The

enzyme reaction was stopped by the addition of 0.1 ml 1 N NaOH. The OD of the solution was obtained by using a Pye-Unicam (SP-500) spectrophotometer at 400 nm. All assays included control tubes without antigen. The amount of antibody detected was assessed by subtracting the OD values of the control tubes from the OD values of the test samples.

Radioimmunoassay. The RIA was performed according to the method described by Gotschlich *et al.* (14). Tyraminated group C meningococcal polysaccharide was obtained from Dr. E. Gotschlich (Rockefeller University, New York) and was labeled with ^{125}I (Amersham) according to the method of Gotschlich *et al.* (14).

Results. Effect of adding BSA (fraction V) to the conjugate solution. Theoretically, it is possible that proteins other than the specific antibodies will adhere to polystyrene surfaces of the tubes not occupied by the specific antibodies, therefore increasing the background staining. To decrease background staining due to this phenomenon, conjugates were diluted in PBS-Tween containing BSA (fraction V) added as a protective blocking agent. Table I shows the effect of addition of BSA fraction V to the conjugate solution. The mean of the OD readings at 400 nm of conjugate controls decreased progressively with increasing concentration of BSA fraction V up to 4%.

These experiments show that the background staining due to the nonspecific reactions is reduced. The no-antigen control, however, still shows some background color. Therefore, this control, representing a nonspecific background reading, was subtracted from the OD readings of all the test sera in the ELISA results.

Specificity of ELISA. To test the specificity of ELISA technique for detecting antibodies to group C meningococcal polysaccharide, we reacted group C polysaccharide coated in tubes with sera known to contain antibodies against the following: Group C meningococcal polysaccharide, group A meningococcal polysaccharide, *Haemophilus influenzae* type b, *Clostridium tetani* toxin, and serum from an individual volunteer with no history of

meningococcal infection (taken as normal serum). The mean OD values observed were 1.36, 0.10, 0.085, 0.07, and 0.09, respectively. These values indicate that it is likely that antibodies to these agents do not cross-react in the ELISA group C meningococcal system.

Reproducibility of ELISA. To evaluate the reproducibility of the assay, we tested three positive sera known to contain antibodies to group C meningococcal polysaccharide 10 times by ELISA over a period of 2 months, during which the sera were stored at 4°. The mean optical density values for sera 1, 2, and 3 were 1.4, 0.92, and 0.72, respectively. The coefficient of

variation for the assay values were 3.2, 1.6, and 4.1, respectively.

Comparison of ELISA and RIA. Following standardization of various ELISA parameters, we compared this method with the RIA. Thirty-three persons from Kafr-Harith Village, Egypt (general population), were chosen at random. Ninety serum samples were obtained from them. These were tested for the presence of group C meningococcal antibodies by both the ELISA method just described and the RIA. These results are shown in Fig. 1. By RIA, 26 of the 90 sera were shown to have antibodies to group C meningococci of more than 5 $\mu\text{g/ml}$ serum. Twenty-two of

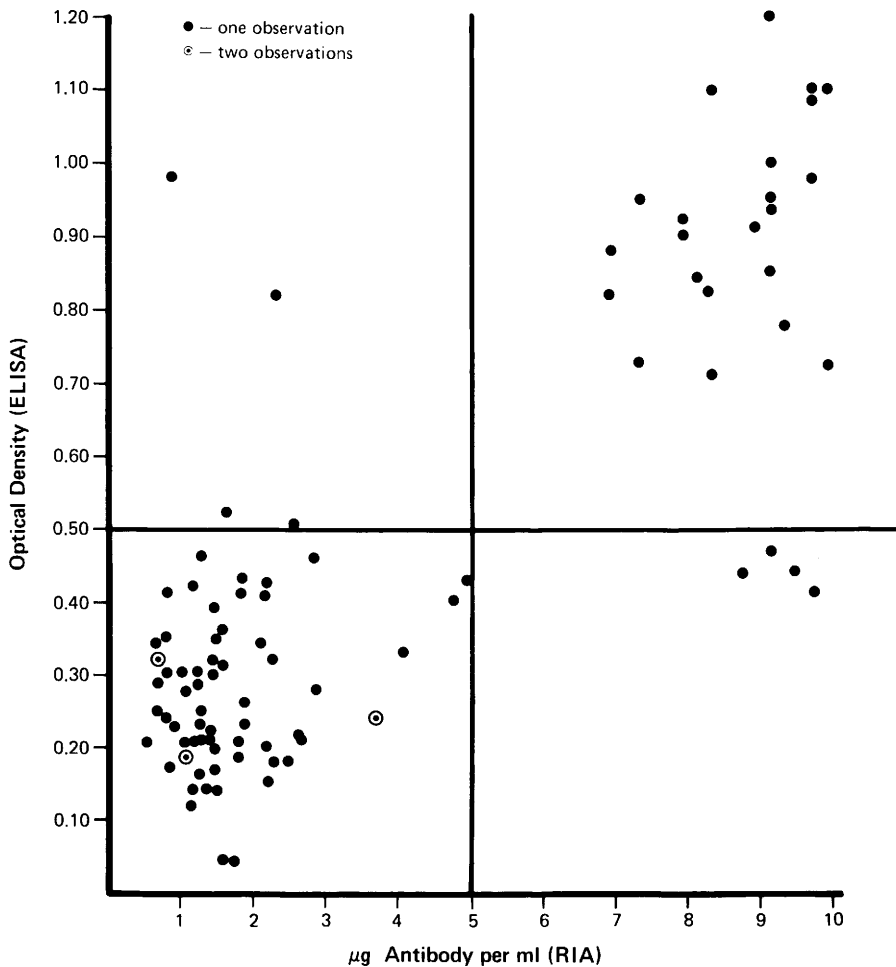


FIG. 1. The group C meningococcal antibody concentrations (μg antibody/ml) as measured by RIA versus ELISA values (OD at 400 nm) for the same sera.

these gave OD readings by ELISA of more than 0.5. Sixty-five sera had antibodies to group C meningococci $< 5 \mu\text{g/ml}$ serum by RIA. With four exceptions, all of the 65 sera gave OD readings of < 0.5 by ELISA. The four exceptions had readings of 0.51, 0.52, 0.82, and 0.98, respectively. Statistical evaluation of these data by correlation coefficient revealed a significant correlation between RIA and ELISA ($r = 0.85$; $P < 0.001$).

Discussion. In the present work a successful method was introduced for indirectly coating polystyrene surfaces with group C polysaccharide through the use of a biospecific binding of the polysaccharide to polystyrene surfaces precoated with IgG fraction of immune group-specific meningococcal antibodies. Following the incubation of polysaccharide antigen, human sera were added and then the conjugated enzyme. The enzymatic color reaction was developed when specific substrates were added. The intensity of color developed was proportional to the amount of antibodies present in human sera directed against the group C meningococcal polysaccharide.

The purity of the different components used in the ELISA proved to be an impor-

tant parameter which affected the non-specific reactions. In addition to purification of the materials used in ELISA, other manipulations were required to minimize nonspecific color reactions. One of these was the addition of 4% BSA fraction V to the dilutions of the conjugates, which resulted in the decrease of the nonspecific reactions (Table I). This supports the use of 4% BSA as reported by Ruitenber *et al.* (15). The background staining still remaining in the case of the no-antigen control (Table I) could be explained as probably due to the presence of nonspecific serum factor(s). Therefore, this nonspecific background reading was subtracted from the OD readings of all test sera in the ELISA results.

The radioimmunoassay is a well-established technique, is highly sensitive, and permits precise quantification of antibodies to meningococcal polysaccharide (14). Our data demonstrated that the ELISA can also be utilized as a sensitive technique for determination of antibodies against group C meningococcal polysaccharide. The RIA has some disadvantages: the use of hazardous, radioactive, nonstable isotopes and the need for expensive equipment. The ELISA is a more convenient method and,

TABLE I. EFFECT OF ADDING BSA FRACTION V TO THE CONJUGATE SOLUTION

Reactions	Percentage of BSA fraction V					
	0	0.5	1	2	4	5
Positive serum control						
Horse anti-c+Ag-c+Ab-c+conjugate+substrate	0.98	0.96	1.10	1.05	1.15	1.15
Negative serum control						
Horse anti-c+Ag-c+Ab-negative-c+conjugate+substrate	0.24	0.24	0.23	0.20	0.18	0.18
Conjugate controls						
Horse anti-c+Ag-c+PBS-Tween+conjugate+substrate	0.16	0.09	0.08	0.07	0.09	0.07
Horse anti-c+PBS-Tween+Ab-c+conjugate+substrate	0.52	0.48	0.38	0.31	0.22	0.21
Horse anti-c+PBS-Tween+PBS-Tween+conjugate+substrate	0.09	0.06	0.04	0.04	0.04	0.02
Mean of conjugate controls	0.25	0.21	0.16	0.14	0.11	0.10

Note. Results are OD at 400 nm.

therefore, should find wide application for measuring antibodies against microbial polysaccharide antigen.

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1. Gold R, Lepow ML, Goldscheider I, Draper TL, Gotschlich EC. Clinical evaluation of group A and C meningococcal polysaccharide vaccines in infants. *J Clin Invest* 56:1536, 1975.
2. Cerebrospinal Meningitis Control. WHO Technical Rep. Series No. 588. Geneva, World Health Organization, 1976.
3. Engvall E, Carlsson HE. Enzyme-linked immunosorbent assay (ELISA). In: Feldman G, ed. First International Symposium on Immunoenzymatic Techniques. Amsterdam, North-Holland, p135, 1976.
4. Leinekki P, Passila S. Solid phase antibody assay by means of enzyme conjugate to anti-immunoglobulin. *J Clin Pathol* 29:1116, 1976.
5. Voller A, Bidwell D, Bartlett A. Enzyme immuno-assay in diagnostic medicine. *Bull WHO* 53:55, 1976.
6. Wisdom EB. Enzyme immunoassay. *Clin Chem* 22:1243, 1976.
7. Wolters G, Kuijpers L, Kacaki J, Schuurs A. Solid phase enzyme-immunoassay for detection of hepatitis B surface antigen. *J Clin Pathol* 29:873, 1976.
8. Yolken R, Kim H, Clem T, Wyatt R, Kalica T, Chanock R, Kapikia AZ. Enzyme immunoassay (ELISA) for the detection of human reovirus-like agent in human stools. *Lancet* 2:263, 1977.
9. Gotschlich EC, Rey M, Etienne J, Sanborn WR, Tria R, Cvjetanovic B. The immunological response observed in field studies of African with group A meningococcal vaccines. *Prog Immunol Standard* 5:485, 1972.
10. Alexander HE, Leidy G, Macpherson CFC. Production of *Haemophilus influenzae* types a, b, c, d, e, and f antiserum for diagnostic and therapeutic purposes. *J Immunol* 54:207, 1946.
11. Flodin P, Killander J. Fractionation of human serum protein by gel filtration. *Biochim Biophys Acta* 63:403, 1962.
12. The TH, Feltkamp TE. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. *Immunology* 18:865, 1970.
13. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA): Quantitative assay of immunoglobulin G. *Immunochemistry* 8:871, 1971.
14. Gotschlich EC, Rey M, Tria R, Sparks KJ. Quantitative determination of the human immune response to immunization with meningococcal vaccines. *J Clin Invest* 51:89, 1972.
15. Ruitenbergh EJ, Steerenberg PA, Brosi BJA, Buys J. Reliability of the enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of *Trichinella spiralis* infections in conventionally raised pigs. *J Immunol Methods* 10:89, 1976.
16. Engvall E, Jonsson K, Perlmann P. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-linked antigen and antibody-coated tubes. *Biochim Biophys Acta* 251:427, 1971.
17. Bruins SC, Ingwer I, Zeckel ML, White AC. Parameter affecting the enzyme-linked immunosorbent assay of immunoglobulin G antibody to a rough mutant of *Salmonella minnesota*. *Infect Immun* 21:721, 1978.

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