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Short communication

ELISA METHODOLOGY FOR POLYSACCHARIDE ANTIGENS: PROTEIN COUPLING OF POLYSACCHARIDES FOR ADSORPTION TO PLASTIC TUBES [1]

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A method is described which permits the adaption of ELISA techniques for measurement of antibody against bacterial polysaccharides. First, the polysaccharide antigen is covalently bound to poly-L-lysine, using cyanuric chloride as the coupling agent. The poly-L-lysine then adsorbs to the walls of plastic tubes, thus immobilizing the polysaccharide coupled to the poly-L-lysine. The method is simple, rapid, and utilizes small amounts of polysaccharide antigen.

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) is now widely used for detection of antibody against protein antigens. It has been difficult, however, to apply the technique for measuring antibody to bacterial polysaccharides. These substances usually carry a net negative charge, due to many acidic groups, and adsorb poorly to plastic and other supporting materials. This problem has been resolved by a method in which the polysaccharide is first bound covalently to a protein which will then adsorb to plastic, thus immobilizing the polysaccharide antigen. The application of this method for measuring antibody to polysaccharide antigens by the ELISA technique is now described.

MATERIALS AND METHODS

Antigens. Bacterial polysaccharides employed included pneumococcus types 6A, 9, 14, and 23 (Dr. Frank Cano, Lederle Laboratories, Pearl River, NY), types 3, 14 and 19 (Dr. James Hill, NIH, Bethesda, MD), and group B

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streptococcus types Ib and II (Dr. Hazel Wilkinson, CDC, Atlanta, GA).

Procedure for coupling polysaccharide antigen to protein for adsorption to plastic tubes. Three test tubes were prepared, containing, respectively (A) 0.5 ml of 0.01 N NaOH with 0.001% phenophthalein indicator; (B) 0.5 mg cyanuric chloride crystals (Aldrich Chemical Co., Milwaukee, WI) and (C) 0.1 ml of 0.1% poly-L-lysine (type VIII; mol. wt. 30,000–70,000; Sigma Chemical Co., St. Louis, MO). Polysaccharide antigens were dissolved in distilled water, 1000 μg/ml. Steps of the procedure are as follows. (1) Alkalinization of polysaccharide: 0.1 ml of antigen was added to tube A and mixed for 10 sec (solution is pink at this stage). (2) 'Activation' by cyanuric chloride: the contents of tube A were pipetted into tube B and mixed for approximately 10 sec, the solution becoming colorless as the pH drops to 8.0–8.2. (3) Coupling to poly-L-lysine: the contents of tube B were pipetted into tube C, mixed, and refrigerated (4°C) for 2 h. (4) Adsorption to plastic tubes: the protein-coupled polysaccharide antigen was then diluted in 0.01 M phosphate-buffered saline (PBS) pH 7.4, dispensed in 1 ml volumes into 2 ml polystyrene autoanalyzer tubes (Elkay). Concentration of antigen for coating plastic tubes was examined with different preparations of protein-coupled polysaccharides (pneumococcal types 3, 6, 14, 19, 23 and group B streptococcal type Ib) diluted to 0.5, 1, 2, and 3 μg polysaccharide per ml PBS (coating solutions). For routine use coating solutions contained 0.75 or 1 μg polysaccharide/ml. Tubes were capped and incubated overnight at room temperature with constant gentle vibration, then stored at 4°C until needed. The maximal antibody binding capacity per tube was determined by ELISA, described below, using specific rabbit antisera at dilutions (usually 10^{-3}) which completely saturate antigenic binding sites.

ELISA technique. ELISA methods were modified from those originally described by Engvall and Perlmann (1972). Serum dilutions and dispensing of anti-antibody conjugates were done by hand; the remainder of the procedure was accomplished with the aid of automated equipment (the 'ELISALYSER') designed and built in this laboratory. (1) Antigen coated tubes were washed for 4 sec with a flow of 18–20 ml deionized water containing 0.05% Brij-35 (Aldrich Chemicals) and suctioned dry. (2) Test sera diluted in PBS-Brij were dispensed in duplicate in 1 ml volumes and incubated for 3 h at 24°C with gentle vibration. Tubes were again washed. (3) 1 ml anti-antibody conjugate (horseradish peroxidase conjugated anti-rabbit IgG, and anti-human IgG; Miles Laboratories, Elkhart, IN), diluted 1 : 500 in PBS-Brij was added to each tube and incubated for 3 h. Tubes were washed. (4) 1.4 ml substrate solution containing 0.005% (1.7 mmol/l) H_2O_2 (USP 3%; Parke-Davis, Detroit, MI) and 0.01% (0.92 mmol/l) *o*-phenylene diamine * (Kodak, Rochester, NY) in PBS was dispensed into each tube and incubated at 24°C for 30 min. (5) Reaction was stopped by

* Kenneth Walls, CDC, Atlanta, GA, personal communication.

the addition of 0.1 ml 4 N NaOH. Absorbance was read at 450 nm with a Brinkman PC1000 fiberoptic spectrophotometer with a 1 cm light path. Duplicate determinations were averaged, and results were calculated as ELISA units, defined as $A_{450}/100$ min. Control tubes consisted of (a) substrate control: antigen coated tubes without serum or conjugate, and (b) conjugate control: antigen coated tubes without serum but *with* conjugate. The substrate control served as a blank in absorbance readings; the conjugate control was used to monitor non-specific adsorption of conjugate, usually <0.02 ELISA units. Non-specific binding or adsorption of IgG in serum was negligible and not routinely monitored. Linearity of the assay was tested using dilutions of whole patient sera and of pooled human gamma-globulin (total IgG approximately 165 mg/ml; Armour Pharmaceutical Co., Phoenix, AZ). The sensitivity of the assay was estimated by testing patient sera previously examined for total specific antibody against pneumococcal type 6, 14, 19 and 23, polysaccharides by Schiffman and Austrian (1971) using a RIA method.

RESULTS AND DISCUSSION

The method for coupling polysaccharides to poly-L-lysine for adsorption to plastic tubes was successful for all antigens tested and permitted adaption of the ELISA technique for detection of specific antibody. Fig. 1 shows

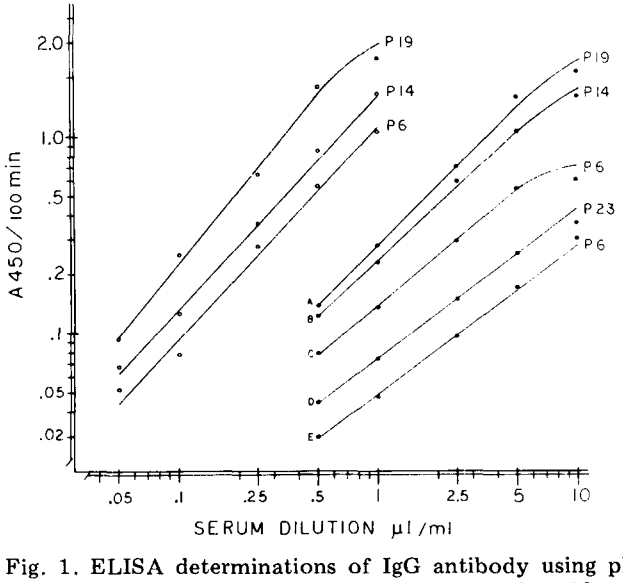

Fig. 1. ELISA determinations of IgG antibody using plastic tubes coated with poly-L-lysine coupled pneumococcal (P) type 6, 14, 19 or 23 polysaccharide were performed on dilutions of pooled human gamma-globulin (○) and patient sera A, B, C, D and E (●). The assay was linear over a 10—20-fold range.

results of ELISA determinations of IgG antibody against pneumococcal type 6, 14, 19 and 23, polysaccharides performed on dilutions of pooled human gamma-globulin and patient sera. The assay was linear over a 10—20-fold range. Above this range, there was increasing competition of antibody for binding sites and the curve began to flatten. The 5 patient sera, A, B, C, D and E, tested by ELISA against pneumococcal types 19, 14, 6, 23 and 6, respectively, contained the following amounts of total homologous antibody nitrogen (AbN)/ml by RIA: A, 201; B, 267; C, 121; D, 200; and E, 50 ng AbN/ml. Based upon detection of pneumococcal type 6 (P6) antibody in 1 μl of serum E (50 ng AbN/ml diluted 10^{-3}), and assuming that antibodies in serum E were predominantly IgG, the estimated sensitivity of the ELISA was at least 0.05 ng AbN/ml per tube. Thus, the linear range of the ELISA was sufficient for testing sera containing 50—500 ng AbN/ml, when diluted 10^{-3}. Suitable dilutions permit measurement above and below this range.

In developing the coupling procedure it was found that 'activation' of polysaccharide (step 2) was best accomplished using an excess of cyanuric chloride crystals, which dissolve and react relatively slowly. In aqueous solution cyanuric chloride reacted so rapidly, giving off HCl, that little coupling could take place before the pH dropped unfavorably. The optimal pH of the alkalinizing solution in tube A (0.01 N NaOH, pH 12) was such that following reaction with cyanuric chloride (step 2) the pH dropped to about 8, which is required for subsequent coupling of amino groups of the protein in step 3. The pH did not usually drop below 8 unless subjected to vigorous mixing (in either step 2 or step 3), or unless the starting pH of the alkalinizing solution was reduced (pH $<$ 12). The most frequent cause of poor coupling, however, was failure to bring the pH below 8.2; the addition of phenophthalein provided a visual indicator of the appropriate pH change. Gelatin was employed as the coupling protein in earlier experiments when developing this method (Gray and Dillon, 1978). Although gelatin coupled antigens performed adequately, the amount of gelatin used (tube C) could vary little from 100 μg/100 μg polysaccharide, and coating solutions (step 4) required at least 1 μg polysaccharide/ml per tube. Poly-lysine coupled equally well at concentrations of 5, 20 and 100 μg/100 μg polysaccharide, and gave more consistent results. Ammonia may be added to block further coupling to unreacted cyanuric chloride, but this was found to be unnecessary because remaining reactive chlorides were hydrolyzed by water in the coating solution.

Reproducibility of poly-lysine coupling and concentration of coupled antigen required for coating solutions was examined in 15 preparations, each represented by a line in Fig. 2. Maximum binding capacity of coated tubes was about 5 ELISA units for 11 preparations depicted by solid lines; antigens included pneumococcal type 3, 6, 14, 19, 23 and group B streptococcal type Ib polysaccharides. Increasing the concentration of polysaccharide antigen above 0.5 μg/ml in the coating solution did not significantly improve binding capacity. Broken lines denote 4 preparations in

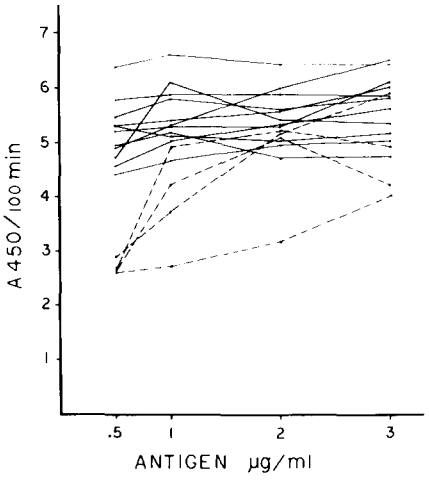

Fig. 2. Reproducibility of poly-L-lysine coupling of polysaccharide antigens and concentration of coupled antigens used for coating plastic tubes (0.5, 1, 2, 3 µg/ml) was examined in 15 preparations of pneumococcal type 3, 6, 14, 19, 23 and group B streptococcal type Ib polysaccharides. Antibody binding capacity in ELISA units (A 450/100 min) was determined by performing the ELISA using rabbit antisera in dilutions which saturate binding sites attached to tubes. Broken lines denote 4 preparations in which poor coupling resulted from failing to allow pH to drop below 8.2 in step 2 of the coupling procedure (see text). 11 normal preparations, denoted by solid lines gave binding capacities of 4—6 ELISA units and showed little difference with increasing concentrations of polysaccharide antigen in the coating solution.

which the pH was not allowed to drop below 8.2 in step 2, as discussed above, resulting in poor coupling. The binding capacity of 3 of these 4 preparations was improved to satisfactory levels by increasing antigen concentration. Adequate binding capacity, 4—6 ELISA units, was equivalent to at least twice the antibody bound at the upper limits of the linear range of the assays shown in Fig. 1 (1—2 ELISA units).

The stability of poly-lysine coupled polysaccharides after storage has not yet been determined. However, gelatin coupled pneumococcal type 3 and 19 polysaccharides retained activity for 1—2 months, while a preparation of gelatin coupled pneumococcal type 23 remained unchanged after nearly a year.

Cyanuric chloride was used by Kay and Crook (1967) to immobilize enzymes on cellulose supports; however, details of the procedure were not given. In the present report, a protein (poly-lysine) was in effect the supporting material for polysaccharide antigens. The protein adsorbs to the walls of plastic tubes, thus immobilizing the polysaccharide covalently bound to the protein. The coupling method is simple, rapid, and utilizes small amounts of antigen. It makes possible the application of ELISA technique suitable for quantitative antibody determination.

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