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Immunospecificity and Quantitation of an Enzyme-Linked Immunosorbent Assay for Group B Streptococcal Antibody

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Type-specific antigen was purified from the supernatant of type III group B streptococcal cultures, tyrosylated, and bound to microtiter wells for an enzyme-linked immunosorbent assay. The immunological specificity of the antigen and the assay was shown by (i) reaction only with homologous unabsorbed rabbit sera and (ii) inhibition after incubation of human serum with homologous but not heterologous purified antigen. The assay was quantitated by relating optical density readings to absolute amounts of human immunoglobulin G bound to the microtiter wells.

The apparent increase in neonatal streptococcal infections in recent years has stimulated considerable interest in human immunity to the group B streptococci. This report describes an enzyme-linked immunosorbent assay (ELISA) for human immunoglobulin G (IgG) antibody to the type III carbohydrate purified from culture supernatants, evidence for the immunospecificity of the assay, and a method for estimating the quantity of IgG detected by the assay.

MATERIALS AND METHODS

Streptococcal strains. R. C. Lancefield provided the prototype group B strains 090 (type Ia), 18RS21 (type II), and D136C (type III). Strain H738 (type III) was isolated from an infant with meningitis and was shown by E. M. Ayoub to produce no detectable extracellular neuraminidase (12).

Rabbit antisera. Absorbed grouping and typing sera were obtained from the Centers for Disease Control, Atlanta, Ga. Unabsorbed hyperimmune serum was prepared in New Zealand albino rabbits by the method of Lancefield (20). Intravenous immunization with formalinized prototype streptococci was continued until the sera demonstrated strong homologous reactions by Ouchterlony double diffusion against hot-acid extracts of the prototype strains.

Analytical methods. Polysaccharide was quantitated by the phenol-sulfuric acid method (14) by using a standard of glucose–*N*-acetylglucosamine–sialic acid–galactose mixed in a molar ratio of 1:1:1:2. This standard represents the composition of types Ia, Ib, and III antigens (4, 18, 19, 28). Protein was measured by the Bio-Rad procedure (Bio-Rad Laboratories, Richmond, Calif.) (10). Counterimmunoelectrophoresis of dilutions of various antigen preparations was done by the method of Fletcher et al. (J. P. Fletcher, G. R. Greene, and E. A. Edwards, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, C155, p. 288) with group- and type-specific sera. Gas-liquid chromatography for monosaccharides was performed by B. M. Gray and D. G. Pritchard (25). Sialic acid was measured in the same laboratory by the thiobarbituric acid assay (31).

Antigen preparation. Preliminary, small-scale experiments showed that strain H738 produced significantly more extracellular type III antigen, estimated by counterimmunoelectrophoresis, than did the prototype strain D136C and indicated the growth and preparative conditions for maximal antigen yield which are detailed below. They also showed that 8 to 64 times more type III antigen could be obtained from culture supernatants than from hot-acid extracts or EDTA-buffer washings (4) of streptococcal cells.

The procedure for large-scale antigen preparation was modified from the preliminary report of Carey et al. (R. Carey, T. K. Eisenstein, G. D. Shockman, and R. M. Swenson, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 18th, Atlanta, Ga., abstr. no. 470, 1978). A dialysate of Todd-Hewitt medium (Difco Laboratories, Detroit, Mich.) was prepared by the method of van de Rijn et al. (29) and was enriched with additional glucose and Na_2HPO_4 to final concentrations of 0.09 and 0.045 M, respectively. The enriched dialysate broth was sterilized by Seitz asbestos filtration (Scientific Products Div., McGaw Park, Ill.) and dispensed to several flasks and carboys. Five streptococcal colonies from a blood agar culture were transferred to 1 liter of dialysate broth which was then incubated overnight and used as the inoculum for a 30-liter culture. The latter was grown as a stationary culture at 37°C for 48 h and harvested with a Sharples continuous-flow centrifuge (Pennwalt Corp., Warminster, Pa.) on loan from Bio-Cell, Inc., Carson, Calif. The pH of the culture supernatant was raised to ≥6.0 with 7 M NaOH, and the supernatant was asbestos filtered. Sodium azide (0.05%) was added to the filtrate and to all buffers to prevent bacterial overgrowth during the lengthy phases of concentration and column chromatography. The culture filtrate was concentrated

to a 10- to 15-ml volume by ultrafiltration through models 2000 and 202 Amicon cells with XM50 membranes (Amicon Corp., Lexington, Mass.). The concentrate was dialyzed against Tris buffer (0.05 M, pH 7.2), and portions were applied to a 100-cm column of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) in Tris and eluted with the same buffer. The eluted fractions containing type or group antigen were pooled, concentrated by Amicon filtration, dialyzed against phosphate buffer (0.05 M, pH 7.4), applied to a column of DEAE-Sephacel (Pharmacia), and eluted with a linear NaCl gradient (0 to 0.3 M) in phosphate buffer. Fractions containing type or group antigen were pooled, concentrated, and stored at −70°C or lyophilized after dialysis against distilled water.

The concentrate of the culture filtrate typically contained carbohydrate and protein in an approximate ratio of 2:1. The type III eluate from Sepharose 4B contained carbohydrate and protein in a ratio of 5:1, and the type III eluate from DEAE-Sephacel was free of detectable protein. The simultaneous application and elution of Dextrans T-2000, T-500, and T-40 (Pharmacia) from Sepharose 4B indicated that the type III antigen had a molecular weight between 5×10^5 and 1×10^6. A typical yield of type III carbohydrate from a 30-liter culture was 5 to 10 mg.

ELISA procedure. Purified type III antigen was tyrosylated by a modification of the method of Insel (R. A. Insel, submitted for publication). A 6.25-mg amount of cyanogen bromide (Mallenkrodt, Inc., St.Louis, Mo.; 125 mg/ml in 0.1 N NaOH) was added to 500 μg of antigen in 1.0 ml of 0.01 N NaOH. The pH of this mixture was maintained at 10.8 with 0.1 N NaOH during a 10-min incubation at room temperature. A 5-mg amount of tyramine hydrochloride (Sigma Chemical Co., St. Louis, Mo.; 50 mg/ml in 0.5 M $NaHCO_3$) was added, and the pH was brought from 10 to 8.5 with 0.1 N HCl. This mixture was dialyzed against distilled water and then against phosphate-buffered saline (PBS) (0.01 M, pH 7.4), each for 24 h at 4°C. Tyrosylated antigen was incubated in wells of Immunolon Removawell microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) for 2 h at 35°C before the addition of serum. All additions to the wells were in volumes of 0.1 ml. A conjugate of affinity-purified, γ-chain-specific goat anti-human or anti-rabbit IgG (Antibodies, Inc., Davis, Calif.) and bovine intestinal alkaline phosphatase (Sigma) was prepared by the one-step glutaraldehyde procedure (2). Each conjugate was titrated in the ELISA system to determine the optimal quantity for use. This was usually 1.0 to 1.5 μg of antibody protein per ml.

Fivefold dilutions of human or rabbit serum in PBS were added to duplicate antigen-coated wells and incubated for 30 min at 35°C. After three PBS washes, the appropriate conjugate was added and incubated overnight at 4°C. After three more washes, *p*-nitrophenylphosphate (1 mg/ml in 10% diethanolamine buffer) was added and incubated in the dark for 1 h at room temperature. The enzymatic reaction was stopped with 3 M NaOH, and the optical density (OD) was determined at 410 nm in a Microelisa Minireader (Dynatech).

Quantitation of ELISA. The amounts of IgG detected in the ELISA were estimated by a modification of the method of Pichichero et al. (24). Normal human IgG (Cappel Laboratories, Cochranville, Pa.) was radioiodinated by a modification of the chloramine T method (23). Twofold dilutions in PBS of labeled IgG (1,000 to 4 ng/ml) were added in triplicate for nonspecific adherence to untreated Immunolon plates. After 2 h at 35°C, the plates were washed three times. The radioactivity of wells was determined in a Biogamma II counter (Beckman Instruments, Inc., Fullerton, Calif.) before and after washing. The goat anti-human IgG conjugate was added, and the ELISA procedure was followed as described above. By selecting points on the linear portion of the curve, the actual concentration of IgG represented by a given OD value was determined from the following equation: actual concentration of IgG = (concentration of IgG added) × (counts per minute after wash/counts per minute before wash).

TABLE 1. Type III ELISA for human IgG with different quantities of purified antigen

Dilution of serum N	OD at 410 nm with the following quantity (μg) of antigen (carbohydrate) added per well:				
	1	0.5	0.25	0.1	0[a]
1:50	1.83	1.83	1.82	1.82	0.08
1:250	1.81	1.82	1.65	1.80	0.03
1:1,250	1.04	1.14	0.79	1.02	0.02
1:6,250	0.32	0.40	0.27	0.38	0.03
1:31,250	0.16	0.17	0.11	0.10	0.01
Buffer	0.02	0.02	0.02	0.01	0.01

[a] In this experiment, the wells had been pretreated with PBS alone. Similar low values were obtained with wells exposed to a dialyzed mixture of cyanogen bromide and tyramine without antigen.

RESULTS

Analysis of purified type III antigen. The high-molecular-weight (5×10^5 to 1×10^6), protein-free, type III antigen eluted from DEAE-Sephacel had the following monosaccharide composition: 31% galactose, 21% glucose, 25% *N*-acetylglucosamine, and 23% sialic acid.

Binding of tyrosylated antigen, IgG, and ELISA conjugates to microtiter wells. Table 1 summarizes an ELISA of dilutions of the serum (serum N) of a normal adult in microtiter wells previously exposed to different concentrations of tyrosylated antigen. Wells precoated with all antigen concentrations tested bound human IgG, presumably specific antibody, in decreasing amounts with increasing serum dilutions. IgG was not bound to wells containing no antigen. This was true whether the wells had been exposed to PBS buffer alone (Table 1, right-hand column) or to the dialyzed mixture of cyanogen bromide and tyramine without antigen (data not shown). Thus, the plastic surfaces of antigen-coated wells did not appear to bind IgG nonspecifically. Similarly, antigen-coated wells treated with buffer but no serum did not appear to bind the ELISA conjugate (anti-IgG) nonspecifically (Table 1, bottom line).

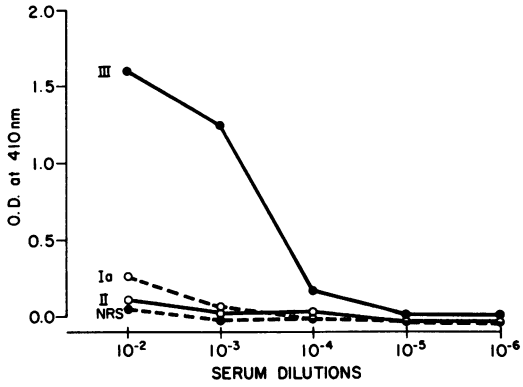

FIG. 1. ELISA for type III antibody in dilutions of unabsorbed and normal rabbit serum. The conjugate contained goat anti-rabbit IgG.

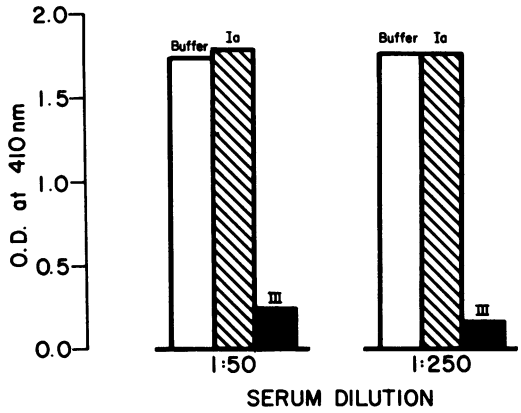

FIG. 2. ELISA for type III antibody after incubation of dilutions of human serum N with purified carbohydrate antigens of types Ia and III.

To conserve the purified type-specific antigen, 0.1 μg per well was selected as the standard quantity for all subsequent assays.

Immunospecificity. Figure 1 represents an assay of unabsorbed sera from rabbits immunized with the prototype group B strains. Type III serum was highly bound in dilutions of 1:1,000 and less, but unabsorbed sera against types Ia and II and normal rabbit serum were not appreciably bound in any dilution tested.

The type specificity was further confirmed by inhibition experiments with purified antigens (Fig. 2). Overnight incubation of dilutions of human serum N with purified type Ia antigen (1 μg/ml) had no effect on the type III ELISA, but preincubation with type III antigen inhibited the ELISA by approximately 85% of the OD value.

Standardization of ELISA assay. Figure 3 represents an assay of human serum N in fivefold dilutions. This serum in these dilutions was then used as a standard for every assay of unknown sera. The latter were tested in dilutions of 1:10, 1:100, and 1:1,000. The dilution of unknown serum which gave an OD value falling on the linear part of the standard curve was selected to determine the level of anti-III IgG relative to serum N.

Figure 4 plots the estimated absolute amounts of IgG represented by different OD values. From this curve, serum N was calculated to contain 14.7 ± 5.6 (standard deviation) μg of anti-III IgG per ml (mean of nine assays).

DISCUSSION

Despite the high level of current interest in the group B streptococci, only two laboratories have measured human antibody to relatively well defined antigens of these streptococci. Both used a radioimmunoassay to type-specific carbohydrates extracted from whole streptococci and purified by chromatographic and other separative procedures (3, 33). Since antibody which is protective for experimental animals and opsonic in vitro is largely type specific (1, 7, 21, 22, 27), studies of this nature are the most promising for understanding and possibly preventing human disease.

In addition to the more obvious advantages of ELISA over radioimmunoassays (greater safety, economy, and possibly sensitivity), the former can be used to measure immunoglobulin class-specific antibody. This advantage is particularly relevant to the question of transplacental immunity (IgG) for the newborn. In this report, we describe the development of an ELISA for human IgG to type III antigen purified from the supernatants of dialysis broth cultures and confirm earlier reports that relatively large amounts of antigen can be prepared from this source (11, 13). Moreover, in molecular weight and chemical composition, our antigen preparation resembled the intact, type-specific carbohydrates of the streptococcal cell wall reported by others (4, 18, 19, 28).

An ELISA for IgG to group B streptococci has been developed in two other laboratories and correlated with other measurements of human antibody (e.g., chemiluminescence as an indication of opsonization of types II and III [26] and mouse protection against type Ia [15]). However, in neither of these reports was the type specificity of the assay rigorously evaluated. In addition, in their earlier reports of a type III radioimmunoassay, Baker and co-workers described a "broad reactivity," or detection of antibody in heterologous, unabsorbed rabbit sera and group B antiserum (3, 5). Our demonstration of significant ELISA binding of only homologous antiserum and of almost complete inhibition of human serum binding by preincuba-

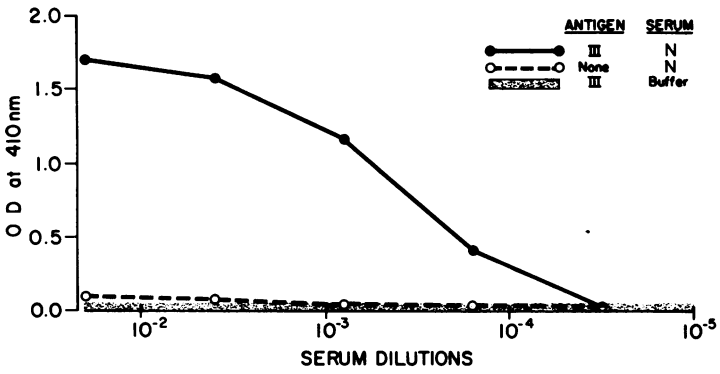

FIG. 3. ELISA for type III antibody of dilutions of human serum N. The open circles represent OD readings when antigen was omitted from the system. The shaded area represents OD readings when human serum was omitted.

tion with purified homologous antigen suggests that the ELISA reported here is essentially free of such cross-reactions.

Several approaches have been developed for coating plastic surfaces with carbohydrate antigens for solid-phase assays, including conjugation of antigen with poly-L-lysine (17) or serum albumin (15) or pretreatment of the surfaces with hyperimmune serum (8). In our experience, tyrosylation of antigen was superior to other methods in promoting a high degree of antigen binding without also causing nonspecific binding of the proteins in test serum or of the anti-IgG ELISA conjugate.

The conventional method of quantitating ELISA and radioimmunoassays has been the quantitative precipitin analysis (16), which measures only precipitating antibody of any immunoglobulin class. In contrast, we attempted quantitation by allowing purified IgG to adhere nonspecifically to untreated microtiter wells and by correlating absolute quantities of IgG per well with ELISA measurements of OD.

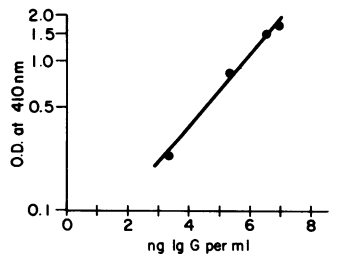

FIG. 4. Quantitation of ELISA. The amounts of human IgG (in nanograms) adhering nonspecifically to microtiter wells was determined from the following formula: amount of adhering IgG = (amount of unlabeled IgG added per well) × (counts per minute in labeled IgG after wash/counts per minute in labeled IgG before wash).

^{125}I-labeled IgG was used to determine the fraction of added immunoglobulin which adhered. Despite theoretical objections (e.g., alteration of the IgG structure during iodination or differences in the configuration of IgG molecules adhering nonspecifically to plastic and binding to antigen by the Fab site), this procedure should provide an accurate estimate of antibody measured by ELISA.

Significant, unresolved questions remain about human immunity to the group B streptococci. For example, the protective role of maternal type III antibody against neonatal infection, reported by Baker and co-workers (3, 5), was not confirmed by Wilkinson, who used a similar radioimmunoassay (32). Moreover, there is considerable uncertainty about the relative prevalence of type-specific immunity in pregnant carriers and noncarriers of group B streptococci (5, 6, 9, 30, 32). Further studies with this assay of sera from carefully studied subjects should help resolve some of these questions.

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