ENZYME IMMUNOASSAY OF THE CAPSULAR POLYSACCHARIDE OF *STREPTOCOCCUS PNEUMONIAE* TYPE III IN CEREBROSPINAL FLUID IN EXPERIMENTAL MENINGITIS

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THE ABILITY to detect microbial capsular antigens that are released from growing organisms into biological fluids has provided a valuable adjunct to the diagnosis of several types of infection (Shackelford, Campbell and Feigin, 1974; Miller *et al.*, 1978). On the other hand, the study of the contribution of capsular antigens to the pathobiology of infectious diseases is limited by the techniques of measurement that are available at present. For example, counter immunoelectrophoresis (CIE), the current method of choice for detection of capsular polysaccharide antigens (Rytel, 1975), is only semi-quantitative and lacks sensitivity for some antigens.

Enzyme immunoassay (EIA) has recently evolved as a quantitative and extremely sensitive method of measuring soluble proteins, including microbial protein antigens (Engvall and Perlmann, 1971; Voller, Bartlett and Bidwell, 1978). It has recently been shown that polysaccharide antigens can also be detected by EIA (Crosson, Winkelstein and Moxon, 1978). There is now a particular need for a more precise method of assay for the capsular polysaccharide of *Streptococcus pneumoniae* because of the renewed interest in the interaction of this antigen with host inflammatory processes (Winkelstein, Bocchini and Schiffman, 1976; Dhingra, Williams and Reed, 1977; Coonrod and Jenkins, 1979) and because of its immunogenical potential (Smit *et al.*, 1977). The present investigation was undertaken to apply EIA to the quantitative study of the *S. pneumoniae* type III capsular polysaccharide in cerebrospinal fluid (CSF) in an experimental model of pneumococcal meningitis in rabbits.

MATERIALS AND METHODS

Experimental model of bacterial meningitis. Meningitis was established in 2-kg New Zealand white rabbits by a previously described technique (O'Donoghue, Schweid and Beaty, 1974). Briefly, rabbits were anaesthetised with sodium pentobarbitone (Abbott Laboratories, North Chicago, IL, USA), 15–30 mg/kg intravenously, and 5×10^6 colony-forming units (c.f.u.) of *S. pneumoniae* type III were inoculated into a marginal ear vein immediately after the injection into the cisterna magna of 0.5 ml of a 0.125% (v/v) suspension of sterile mucin (Sigma Chemical Co.,

Received 20 Feb. 1980; accepted 6 May 1980.

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St Louis, Mo, USA). Seventy-five per cent of rabbits handled in this manner develop pneumococcal meningitis and die within 3–6 days if untreated. Animals selected to receive antimicrobial therapy for their infection were given penicillin G, 150 mg/kg intramuscularly every 12 hours for 5 days.

For control purposes, five rabbits were anaesthetised and given an intracisternal injection of 0.5 ml of a suspension containing 10^7 – 10^8 c.f.u. of the following bacteria: *Haemophilus influenzae* type b (three rabbits), *S. pneumoniae* type XXV (one rabbit), and *S. pneumoniae* type IIIR (an unencapsulated variant kindly provided by Dr Robert Austrian) (one rabbit).

Broth cultures of pneumococci. Several strains of pneumococcus were cultured in trypticase soy broth supplemented with 5% bovine albumin. At defined intervals, portions were removed for determination of the concentration of bacteria by culture of serial dilutions in poured agar plates, and for assay of type-III pneumococcal polysaccharide by EIA and, in some instances, by CIE. Strains tested in this manner were the type-III pneumococcus used to produce the experimental infection in rabbits, an attenuated variant of this strain produced by serial passage on blood agar for 3 months, and the following *S. pneumoniae* strains from the American Type Culture Collection (ATCC), Rockville, Md, USA: 6301 (type I), 6303 (type III), 6312 (type XII) and 6325 (type XXV).

Collection and preparation of specimens. CSF was obtained from control and infected rabbits by puncture of the cisterna magna with a 25-gauge needle after pentobarbitone anaesthesia. Specimens visibly contaminated with blood were rejected. Bacteria were enumerated by culture of serial dilutions of CSF in poured agar plates. CSF specimens were then centrifuged at 3000 *g* for 10 min., a procedure that had been shown to remove more than 99% of bacteria and white blood cells from the supernates which were then stored at -70°C until assay by EIA or CIE, or both. On a day of assay, a CSF specimen was thawed and 0.2 ml was pipetted into a disposable 12 $\times$ 75-mm plastic tube. To this 1.8 ml of 0.01M phosphate buffer, pH 7.2, were added. Then, at least six sequential two- or threefold dilutions of this tenfold diluted CSF specimen were prepared in the same buffer. All dilutions were added to the EIA in 1 ml portions. Broth cultures of pneumococci were handled similarly: supernates were prepared and stored at -80°C , thawed on the day of study, and diluted tenfold initially, then subsequently in six twofold or threefold sequential steps. All dilutions were assayed. Standard solutions of capsular purified pneumococcal polysaccharide (Merck Sharp & Dohme lot no. 3950-3K, kindly supplied by Dr John B. Robbins, Bureau of Biologics, Bethesda, Md, USA), were prepared by dissolving a known weight of the solid material in 0.01M phosphate buffer, pH 7.2, or supplemented TSB. These standard solutions were diluted appropriately with phosphate buffer and added to EIA preparations in 1-ml portions.

Preparation of reagents. Rabbit antiserum to formalin-treated type-III *S. pneumoniae* was purchased from the Division of Laboratories and Research, New York State Department of Health, Albany, NY (Dr Kenneth Amiraian). The immunoglobulin fraction of this antiserum was prepared by precipitation with Na_2SO_4 as described by Voller, Bidwell and Bartlett (1976). This immunoglobulin (antiserum-globulin) was lyophilised and stored at -20°C until used. Antiserum-globulin, 5 mg, was conjugated with an equal amount of horseradish peroxidase (Sigma) by the method of Nakane and Kawaoi (1974). This technique permits the linkage of carbohydrate moieties of the enzyme to free amino groups of the immunoglobulin; the result is efficient production of enzyme-immunoglobulin conjugates and a minimal amount of immunoglobulin-immunoglobulin conjugation. This procedure produced approximately 3 ml of a golden-coloured solution (antibody-enzyme conjugate) which was stored at 4°C in 0.01% (w/v) sodium azide (Fisher Scientific Co., Fair Lawn, NJ, USA) until it was used. Orthophenylenediamine (Eastman Kodak Co., Rochester, NY) was dissolved in distilled water at a concentration of 100 mg/litre. To this was added 3% (v/v) hydrogen peroxide (Fisher) in an amount sufficient to bring its concentration to 0.3%. The solution ($\text{OPD-H}_2\text{O}_2$) was freshly prepared daily for each experiment.

EIA procedure. A single-antibody sandwich technique was used to assay the type III pneumococcal capsular polysaccharide. First a known weight of the lyophilised antiserum-globulin was dissolved in carbonate buffer, 0.05M, pH 9.6. One-ml amounts of this antiserum-globulin solution were added to 12 $\times$ 75-mm polystyrene tubes and incubated at 37°C for 4 h.

The tubes were then emptied and washed three times with 1 ml of 0.06% (w/v) polyethylene sorbitan monolaurate (PSM, Fisher) in 0.02M phosphate buffer, pH 7.2. After this wash, which was also performed after each subsequent step, 1-ml portions of solutions of purified pneumococcal type-III polysaccharide, test specimens of CSF, or broth culture supernates were added to the tubes and incubated for 2 h at 37°C. The tubes were then emptied and washed. Next, the original antibody-enzyme conjugate solution was diluted in the phosphate-buffered PSM, added to the tubes in 1-ml amounts, and incubated for 2 h at 37°C. Finally, after another wash, 1 ml of the OPD-H₂O₂ solution was added to each tube and incubated for 1 h in the dark at room temperature. The reaction was stopped by adding to each tube 0.5 ml of 8M H₂SO₄. In this enzyme-substrate reaction, the colourless substrate OPD is converted to a compound with a golden colour, the intensity of which is proportionate to the amount of antibody-bound enzyme on the inner surfaces of the tubes. The optical density of these solutions at 490 nm was determined in a spectrophotometer (Spectronic 20, Bausch & Lomb, Rochester, NY) fitted with a semi-automatic flow-through cuvette (Bausch & Lomb) and recorded as percentage transmittance (%T).

Counterimmuno-electrophoretic determinations. Quantitative counter-immuno-electrophoresis was performed in plates of 1% agarose (Sigma) according to a previously described procedure (Coonrod and Rytel, 1973), that involves assay of serial dilutions of standards and unknowns. Reagents (undiluted rabbit type-III antiserum and the purified type-III pneumococcal polysaccharide or CSF supernates) were added in 10- μ l portions to paired wells cut 0.3 cm apart in the agar; the preparations were run for 1 h at a current of 30 mA (electrophoresis chamber, MRA Corp., Clearwater, Fl, USA) in 0.05M tris (hydroxymethyl) aminomethane-barbitone buffer, pH 8.6. Precipitin arcs were identified by visual examination with a strong oblique source of light.

RESULTS

Optimum conditions for the assay of the type-III pneumococcal capsular polysaccharide by EIA were established by block titration. In this process the concentration of antiserum-globulin in the initial coating step was varied from 0.1 μ g/ml to 10 μ g/ml, and the original antibody-enzyme conjugate solution was tested in dilutions from 2×10^2 to 8×10^3 . Fig. 1 shows the results of an EIA of twofold dilutions of a 10 ng/ml standard solution of the type-III pneumococcal capsular polysaccharide with the use of 1.0 μ g/ml antiserum-globulin to coat the tubes and a 4×10^3 dilution of the antibody-enzyme solution. The dose-response curve demonstrates a log-linear change in optical density with sequential dilutions in the range of polysaccharide concentrations from 1.25 ng/ml to 0.16 ng/ml. These were the conditions chosen for the assay of test specimens of CSF and this standard curve was developed with each daily experiment. Concentrations of the polysaccharide antigen in unknown specimens of CSF or in broth were determined by diluting each specimen so that at least one reading fell on the linear portion of the standard curve. Specimens with optical density below the linear portion of the curve were declared to be negative. Reproducibility of the assay was excellent. The mean ($\pm$ one standard deviation) of 10 consecutive determinations of the 0.625 ng/ml standard pneumococcal polysaccharide solution 3 months after preparation of the reagents was 33.8 ± 2.2 %T; 6 months later that corresponding value for 10 consecutive determinations was 51.8 ± 3.1 %T. At that latter time the original level of sensitivity could be restored by the use of a 3×10^3 rather than a 4×10^3 dilution of the original antibody-enzyme solution.

FIG. 1.—Dose-response curve for the enzyme immunoassay of purified type-III pneumococcal capsular polysaccharide in 0.01M phosphate buffer, pH 7.2. The tubes were coated initially with antiserum-globulin 1 μ g/ml and the antibody-enzyme conjugate was used in a 4×10^3 dilution of the original solution.

Reproducibility of the assay was also assessed by periodically assaying four replicates of an unknown CSF specimen. The standard deviations were always less than 5% of the means. Likewise, results of assays done on different days of portions of a single unknown CSF specimen agreed within 10% if performed within 3 weeks.

By using the EIA technique, we could detect type-III capsular polysaccharide in broth culture supernate very early during growth of type-III pneumococci (table I). Concentration of the capsular antigen in broth increased proportionately with the increase in concentration of bacteria in the culture. By contrast, the capsular polysaccharide was not detected by quantitative CIE until the organism entered the logarithmic phase of growth and reached a concentration in broth greater than 10^6 c.f.u./ml.

The specificity of this EIA technique for the pneumococcal type-III capsular polysaccharide was evaluated by the assay of supernates of overnight broth cultures of several capsular types of pneumococci (table II). Types XII and XXV pneumococci showed no cross reactivity and a type-I strain showed a minimal amount. The type-III pneumococcal strain used to produce experimental meningitis elaborated more than twice as much capsular polysaccharide into the broth as an ATCC type-III strain. However, the experimental

TABLE I

Amount of capsular polysaccharide discharged into broth medium during growth of type-III pneumococci*

Hours of incubation	Concentration of pneumococci (c.f.u./ml)	Concentration of polysaccharide (ng/ml) by	
		EIA†	CIE
2	4×10^2	15.6	< 50
4	8×10^2	19.0	< 50
8	6×10^4	60.0	< 50
12	6×10^6	5438	2000

EIA = enzyme immunoassay; CIE = counterimmunoelectrophoresis.

* Trypticase soy broth with 5% bovine albumin.

† Uninoculated broth was negative by EIA at all times.

pneumococcal strain, when attenuated by serial passage on blood agar, discharged very little polysaccharide into the broth (table II).

According to EIA testing of CSF from rabbits with pneumococcal meningitis, the concentration of the capsular polysaccharide increased progressively as meningitis developed (fig. 2). This increase closely paralleled that of the mean CSF concentration of bacteria during the same time period, from 5.5×10^4 c.f.u./ml at 24 h to 2.3×10^6 c.f.u./ml at 96 h (data not shown in fig. 2). Each CSF specimen from rabbits with meningitis contained levels of the capsular polysaccharide well above the lower limit of sensitivity of EIA; those from uninfected rabbits were always negative. Those CSF specimens were all tested by CIE (lower limit of sensitivity, 50 ng/ml): two of the four 24-h specimens were positive, as were four of the six 48-h specimens and five of the six 72-h specimens. Each 96-h specimen was positive by CIE.

CSF of five rabbits injected intracisternally, 24 h previously, with *H.*

TABLE II

*Enzyme immunoassay of type-III capsular polysaccharide in broth during culture of several strains of pneumococcus**

Pneumococcal strain	Type	Concentration of polysaccharide (ng/ml)
Experimental†	III	26 250
Attenuated‡	III	67.5
ATCC6301	I	1.6
ATCC6303	III	12 000
ATCC6312	XII	< 1.0
ATCC6325	XXV	< 1.0

* Each strain was cultured in trypticase soy broth with 5% bovine albumin for a time sufficient to produce a count of approximately 5×10^7 c.f.u./ml.

† Strain used to produce experimental meningitis in rabbits.

‡ Strain produced by serial passage of the experimental strain on blood agar for 3 months.

FIG. 2.—Concentration of the type-III capsular polysaccharide in cerebrospinal fluid of rabbits with experimental pneumococcal meningitis as measured by enzyme immunoassay.

influenzae type b or *S. pneumoniae* types XXV or IIIR were all negative when assayed for the type-III pneumococcal polysaccharide.

Successful antimicrobial therapy of pneumococcal meningitis with penicillin G was associated with a rapid decrease in the concentration of the capsular polysaccharide in CSF (fig. 3). The antigen, however, was still detected in CSF of all rabbits after 4 days of effective therapy. Moreover, the concentration of the capsular polysaccharide remained elevated in one animal whose infection persisted during therapy, and increased in concert with the increase in the CSF concentration of pneumococci.

DISCUSSION

The present study confirms recent reports which show that the polysaccharide capsular antigens of several pathogenic bacteria can be detected by EIA in concentrations as low as 1–10 ng/ml in CSF (Crosson *et al.*, 1978; Drow, Maki and Manning, 1979; Harding *et al.*, 1979). This level of sensitivity is equal to that of radioimmunoassay (O'Reilly *et al.*, 1975) and is considerably greater than that of CIE, the current method for assay of polysaccharide antigens (Rytel, 1975).

Like others, we used a sandwich EIA technique in which the globulin fraction of type-specific pneumococcal antibody was adsorbed to the solid phase before antigen attachment. This step appears necessary for optimal

FIG. 3.—The response of pneumococcal capsular polysaccharide in cerebrospinal fluid (CSF) during treatment of meningitis with penicillin G 150 mg/kg intramuscularly every 12 h. The actual range of antigen concentrations in CSF at initiation of therapy was 157.5–25 000 ng/ml. The animal that was culture positive at 48 and 96 h had an initial antigen concentration of 500 ng/ml and a 96-h value of 581.3 ng/ml. ○—○=Results from one culture-positive animal; ●—●=mean of results from eight culture-negative animals.

assay of polysaccharide antigens because these substances are not adsorbed well when applied directly to polystyrene, the material usually used as the solid phase for EIA techniques. To enhance further the sensitivity of EIA, some workers have advocated the use of an indirect-antibody technique (Engvall and Perlmann, 1972) that avoids enzyme labelling of the antigen-specific antibody and possible loss of activity. We found, however, that conjugating the enzyme directly to the type-specific pneumococcal antibody deleted one step from the assay without apparent loss of activity. This finding may be attributable to the improved method of conjugation recently described by Nakane and Kawaoi (1974).

Recent reports of detection of bacterial capsular antigens by EIA emphasise the diagnostic potential of the assay (Crosson *et al.*, 1978; Drow *et al.*, 1979; Harding *et al.*, 1979). In contrast, the EIA method reported here is designed for more quantitative study of the pneumococcal capsular polysaccharide. Our results indicate that EIA should facilitate study of the pathogenesis of bacterial meningitis and the response of the central nervous system during that infection. For example, EIA offered an interesting perspective on an old observation regarding virulence of pneumococci. The type-III strain used to produce meningitis has been inoculated into and recovered from CSF of rabbits continually for more than 10 years. This strain discharged during

growth in broth culture more than twice as much soluble capsular material as that elaborated by a type-III strain obtained from the ATCC. Moreover, the experimental strain lost much of its potential for polysaccharide production after passage on blood agar for three months (table I). During this time the organism also lost considerable virulence for producing meningitis in rabbits (unpublished observations). These observations interpret, in quantitative terms, the known relationship between the virulence of pneumococci and the extent of their capsules (Enders, Shaffer and Wu, 1936; Austrian and Gold, 1964).

Knowledge of the range of capsular antigen concentrations in CSF should also clarify the role of the pneumococcal capsular polysaccharide as a reactant with host inflammatory processes. The frequently quoted "antiphagocytic" property of the type-III pneumococcal capsule (Wood and Smith, 1949) appears to be a dose-dependent phenomenon (Winkelstein *et al.*, 1976; Dhingra *et al.*, 1977) and the concentrations of the purified capsular polysaccharide necessary to produce this effect *in vitro* are far greater than those measured in CSF in the present study. Likewise, large concentrations of purified type-III pneumococcal polysaccharide activate complement via the alternative pathway, but this effect diminishes as the concentration of polysaccharide is reduced to 1 µg/ml (Giebink *et al.*, 1978; Coonrod and Jenkins, 1979).

It remains to be determined whether the pneumococcal capsular antigen in concentrations anticipated in CSF acts as the chemotactic factor that stimulates the influx of granulocytes into CSF in experimental pneumococcal meningitis (Nolan, Clark and Beaty, 1975), or whether it contributes to the CSF metabolic acidosis that supervenes terminally in that infection (Sears *et al.*, 1974). EIA should contribute to the clarification of these and other important unresolved issues relating to the pathogenesis of bacterial meningitis and its morbidity.

SUMMARY

An enzyme immunoassay (EIA) for the capsular polysaccharide of *Streptococcus pneumoniae* type III was developed and applied to the measurement of this antigen in cerebrospinal fluid (CSF) in an experimental model of pneumococcal meningitis. EIA was performed by a single-antibody sandwich technique in which the globulin fraction of pneumococcal type-specific antiserum (antiserum-globulin) was used to coat the solid phase before antigen attachment and to conjugate with the labelling enzyme, horseradish peroxidase. Under optimum assay conditions EIA detected purified pneumococcal type-III capsular polysaccharide in concentrations as low as 0.15 ng/ml in aqueous buffer. Assayed by EIA, the mean concentration of type-III capsular polysaccharide in CSF of rabbits with pneumococcal meningitis increased exponentially from 24 h to 96 h of infection (range 13.9 ng/ml–62 500 ng/ml). Effective antimicrobial therapy of rabbits with meningitis was associated with a rapid decrease in the CSF concentration of the capsular antigen, although it was still detected in concentration in the range 1–10 ng/ml in 100% of animals treated

for 4 days. Thus EIA provides a quantitative and extremely sensitive method of measuring type-III pneumococcal capsular polysaccharide in CSF.

This work was supported by NIH-PHS Biomedical Research Grant SO7RR05350. We are grateful for the expert secretarial services of Mrs Inelle Reynolds.

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