

# Circulating Polyribophosphate in *Hemophilus influenzae*, Type b Meningitis

## CORRELATION WITH CLINICAL COURSE AND ANTIBODY RESPONSE

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**ABSTRACT** In systemic infections caused by *Hemophilus influenzae*, type b, the capsular polysaccharide, polyribophosphate, is released into the circulation. Polyribophosphate was quantitated in serial serum and cerebrospinal fluid samples from 45 children with *H. influenzae*, type b meningitis by means of a radiolabeled antigen-binding inhibition assay. Polyribophosphate was regularly found in acute serum and cerebrospinal fluid samples and could be detected in unbound form for periods of 1–30 days after initiation of effective therapy. Complexes of polyribophosphate dissociable with acid and pepsin were detected in serum samples from 17 patients, in one case for a period of 145 days after hospitalization. Polyribophosphate levels and patterns of clearance were studied in relation to hospital course and antibody response. Patients with prolonged antigenemia had protracted fevers and severe neurological symptoms during hospitalization, frequently with focal complications. Antipolyribophosphate antibody responses were detected during the first 100 days of convalescence by radioimmunoassay in 79% of the patients studied, including 60% of the children 1 yr or less in age. The intensity of antibody response although clearly related to the age of the patient, was more reliably predicted by the efficiency of antigen clearance. Antibody responses were uniformly of low magnitude in patients with prolonged antigenemia, irrespective of age. Patients who failed to develop antibody to polyribophosphate after meningitis also exhibited impaired antigen clearance. These studies suggest that mechanisms necessary for clearance of polyribophosphate may influence

the development and intensity of the humoral immune response and raise the possibility of developmental deficiencies in the clearance system in infants and children.

## INTRODUCTION

Evidence derived from several decades of clinical and experimental investigation of *Hemophilus influenzae*, type b have led to the identification of the capsular polysaccharide, polyribophosphate, (PRP)<sup>1</sup> as the critical virulence factor in systemic infection, and have demonstrated that passive immunization with antibody to this antigen is effective in the prevention and treatment of such infections (1). Seroepidemiologic surveys have shown that the human immune response to clinical infection and to asymptomatic carriage of the organism usually includes the development of antibodies to the PRP component (2, 3). Due in part to interest in whether active immunization with PRP might be protective, recent studies have examined the response to infection with particular interest in the anti-PRP antibodies produced in children ½–3-yr old, the age group most susceptible to systemic infections. Thus, Schneerson et al. found antibody responses measurable by passive hemagglutination in all of 45 patients with systemic infections, including 11 children in the 1st and 5 in the 2nd yr of life (4). Using a similar technique however, this laboratory and others have found antibody responses to be frequent in older children but rare in those under 2 yr of age (5–7). Based on these observations, Norden et al. suggested the younger children might be immunologically paralyzed (6).

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<sup>1</sup> Abbreviations used in this paper: CIE, countercurrent immunoelectrophoresis; CSF, cerebrospinal fluid; PRP, polyribophosphate.

PRP is released from the bacilli during infection, and immunological detection of the antigen in cerebrospinal fluid (CSF) has been used for rapid diagnosis of meningitis (1, 8-11). Elevated concentrations of PRP in the CSF at admission have been found to be prognostic of a difficult clinical course (10, 12).

The present study has employed tritium-labeled PRP in the development of assays of antibody and antigen more sensitive than heretofore used with infected patients. These techniques have been used to study the elaboration and clearance of circulating PRP during the course of infection, and to examine the relation of antigenemia to the clinical course and to the antibody response. Further study of the relation of antibody response to the age of the infected child was deemed worthwhile for resolution of the apparent disagreement in published observations mentioned above and for its possible relevance to the feasibility of active immunization with PRP in infancy.

## METHODS

45 of the 70 children admitted to the Children's Hospital Medical Center with *H. influenzae* b meningitis in 1970-1972 comprised the study population. In each case, the diagnosis was made by serologic typing of the *H. influenzae* isolated from CSF or blood. Ampicillin was administered intravenously, usually in a dosage of 300 mg/kg per day, for at least 10 days or until clinical parameters of infection and abnormal CSF findings had resolved. Signs and symptoms of each patient before hospitalization, at admission, and during the hospital course were summarized. Specifically noted were fever, neurologic and cardiovascular symptoms, the presence of subdural effusions and potential chronic foci of infection, such as sinusitis, mastoiditis, arthritis or pneumonia; chemical and cytologic abnormalities of the CSF and of blood were also recorded. A patient was judged to have a severe presentation if he was admitted in a coma or stupor, was responsive only to deep pain, or was in status epilepticus or shock. A patient's hospital course was defined as severe if marked by coma of more than 24 h duration, recurrent or poorly controlled seizures, cranial or peripheral motor nerve deficits, ataxia or deafness persisting throughout hospitalization, periods of apnea, or subdural effusions requiring multiple taps or surgical intervention.

**Clinical samples.** CSF samples used for PRP assays were collected aseptically; cells and bacteria were sedimented by centrifugation for 2.5 min in an Eppendorf centrifuge at 8,000 g. The cell-free supernate was stored in sterile containers at  $-70^{\circ}\text{C}$  without preservatives. Blood samples were obtained by venipuncture and the serum stored as above. Sequential serum samples were obtained from all children when feasible, but volumes did not always suffice for all analyses; thus the number of observations presented may be fewer than the total number of patients. Urine samples were collected without preservative and were stored as above.

**Immunological reagents and methods.** The PRP preparation employed has been described elsewhere (13). Anti-PRP antibody activity was determined by a double-label modification (14) of the radioantigen-binding technique of

Farr (15), using  $^3\text{H}$ -labeled PRP and  $^{36}\text{Cl}^-$  as a volume marker. Details of the technique have been described (16).

Briefly, PRP was intrinsically labeled by culturing *H. influenzae* type b, in the presence of  $[\text{}^3\text{H}]$ glucose. The purified labeled PRP had a specific activity of approximately  $5 \times 10^4$  dpm/mg. For the weakest sera, this antigen was used at a concentration of 6.3 ng/ml in phosphate-buffered saline. To accurately estimate higher antibody levels,  $[\text{}^3\text{H}]$ -PRP at this concentration, was mixed with unlabeled PRP to give standard solutions containing 6.3, 20, 74, and 700 ng/ml PRP.  $\text{Na } ^{36}\text{Cl}$  was added to a concentration of 0.15  $\mu\text{Ci/ml}$  as a volume marker. 25  $\mu\text{l}$  of antigen solution was mixed with 50  $\mu\text{l}$  of test serum in a small conical polypropylene centrifuge tube and incubated 4 h at  $4^{\circ}\text{C}$ . 75  $\mu\text{l}$  of saturated ammonium sulfate was added; incubation was continued 1 h, the mixtures were centrifuged and the supernate removed, all at  $4^{\circ}\text{C}$ . The precipitate was dissolved with water and suspended by the use of Protosol (New England Nuclear, Boston, Mass.) in a toluene-based scintillation fluid. A liquid scintillation spectrometer was adjusted so that one channel counted  $^{36}\text{Cl}$  at an approximate efficiency of 75% and  $^3\text{H}$  at 0.03% and the second channel counted  $^{36}\text{Cl}$  at 1.5% and  $^3\text{H}$  at 15% efficiency. Calculation of the percentage antigen-bound was done as described by Gotschlich (14). To determine the antibody concentration, a reference human serum supplied by Dr. J. B. Robbins, whose precipitin content is known, is initially tested in serial dilution against each of the standard concentrations of PRP. Plotting percent of  $[\text{}^3\text{H}]$ PRP bound vs. dilution of this serum yields a series of standard curves. The test serum is assayed undiluted with the series of antigens. The percent binding, obtained from the most appropriate antigen concentration, is then equated to a dilution of the reference serum, and thus to a known antibody concentration. The reaction is specific. Binding of the radiolabel may be completely inhibited by addition of unlabeled PRP or by absorption of sera with insolubilized PRP or with *H. influenzae* b. Conversely, absorption with unencapsulated mutants of *H. influenzae*, does not reduce the observed antibody concentration. The limit of detection with 50- $\mu\text{l}$  serum samples was 6 ng antibody/ml serum; concentrations of  $< 7$  were calculated as 7 ng/ml. A significant change in an individual's antibody concentration was defined as an increase exceeding the  $t_{0.05}$  confidence interval determined for replicate samples in the assay.

PRP in clinical specimens was quantitated by a variation of the antibody assay in which the inhibition of binding of  $^3\text{H}$ -labeled PRP by a standard amount of antibody is assayed. In order also to detect PRP complexed with antibody of other proteins, a modification of a technique suggested by Dr. Gerald Shiffman was employed.<sup>2</sup> A 25- $\mu\text{l}$  sample of test serum or CSF is incubated at  $56^{\circ}\text{C}$  for 2 h with an equal volume of 0.1 M citric acid, pH 2.0, containing 1 mg pepsin/ml (Worthington Biochemical Corp., Freehold, N. J.), after which the digest is neutralized with 50  $\mu\text{l}$  of 0.25 M  $\text{Na}_2\text{HPO}_4$  and cooled on ice to inactivate the pepsin. In parallel, a matched 25- $\mu\text{l}$  test sample is initially incubated with 75  $\mu\text{l}$  of a neutralized pepsin solution, containing 1 vol of the citric acid-pepsin mixture and 2 vol of 0.25 M  $\text{Na}_2\text{HPO}_4$ , for 2 h at  $20^{\circ}\text{C}$ . The digested and undigested samples are then incubated 2 h at  $4^{\circ}\text{C}$  with 25  $\mu\text{l}$  of a standard anti-PRP preparation and then overnight with 25  $\mu\text{l}$  of  $^3\text{H}$ -labeled PRP (5 ng/ml) containing  $^{36}\text{Cl}^-$ . The antibody is precipitated at 50% saturation with  $(\text{NH}_4)_2\text{SO}_4$ , and separated by centrifuga-

<sup>2</sup> Shiffman, G., personal communication.

tion. The precipitate is solubilized, and the radioactivity of the bound [ $^3\text{H}$ ]PRP counted as previously described (16). Human serum shown to be free of PRP or anti-PRP antibody serves as a negative standard. Inhibition of antigen binding is calculated according to the formula:

$$\frac{A - B}{A} \times 100 = \text{percent inhibition,}$$

where A = percent [ $^3\text{H}$ ]PRP bound in the negative control, and B = percent [ $^3\text{H}$ ]PRP bound in the test sample. Purified PRP diluted in normal serum to known concentrations is similarly tested to establish a standard curve, from which the concentration of PRP in test specimens is estimated. The test is sensitive to PRP concentrations as low as 0.5 ng/ml when a standard [ $^3\text{H}$ ]PRP, added at a concentration of 5 ng/ml, is used. With duplicate assays, differences in PRP concentration as low as 1.5 ng/ml are significant at the 99% confidence level. The concentration of PRP estimated in the digested sample is a measure of total PRP, and in the undigested sample, free PRP; the difference is thus an estimate of complexed PRP.

The digestion technique was calibrated with PRP-human anti-PRP antibody complexes made in vitro. PRP can be quantitatively recovered from such complexes over a wide range of antigen-antibody ratios. Citric acid at 56°C is alone sufficient to release PRP from complexes with antibody (pepsin merely aids solubilization); this is also true of the complexed PRP in patients' sera. At present, however, we have no direct evidence that the complexing agent in such sera is antibody.

*Statistical methods.* Means, variances, linear regressions, correlation coefficients, confidence intervals, and significance tests were performed with formulas entered into a Wang 360K programmable calculator (Wang Laboratories, Inc., Tewksbury, Mass.). The equations for statistical tests and statistical tables were obtained from two sources (17, 18).

## RESULTS

*PRP in body fluids.* Of specimens obtained on admission, free PRP was detected in the serum of 34 of 38 cases, and in 11 of 12 CSFs. These CSFs included samples from two of the four patients without detectable PRP in the serum; both were positive. The one patient lacking detectable PRP in his CSF had a serum concentration of 500 ng/ml PRP, consistent with a culture-documented bacteremia. Thus, PRP was detectable in either serum or CSF in each of the 36 patients suitably studied. The concentration of PRP in serum and CSF samples ranged from 1-1,100 ng/ml, with median concentrations of 25 ng/ml in serum and 28 ng/ml in CSF. While the CSF level exceeded that in matched serum samples in 7 of the 10 cases studied, the ratio of CSF/serum PRP concentrations varied from 0.001 to 238.

Free PRP was detectable in serum for up to 30 days after hospitalization. The antigen was detected only on hospital day 1 in 27 children, in 5 children for 2-9 days, in 9 for 10-19 days, and in 2 for 20 or more days.

One determinant of the duration of antigenemia was the serum concentration of PRP attained in the course of infection. Patients with antigenemia for 3 or more days after hospitalization had higher concentrations of PRP in their admission serum (mean = 60 ng/ml) than the patients effecting clearance during the first 2 days of hospitalization (mean = 4.3 ng/ml). This difference was quite significant ( $P < 0.0005$ ) but not absolute, since two patients with relatively low levels of PRP initially (< 40 ng/ml) exhibited delayed antigen clearance.

The kinetics of antigen clearance exhibit at least two patterns suggesting that additional factors affect antigen elimination. As shown in Fig. 1, the concentration of antigen declined exponentially during the first 2 hospital days in all but two of the cases. The rate of decline, however, was distinctly slower in the group of patients effecting delayed clearance. In one patient, an initial precipitous decline in PRP concentration was followed by a slow resolution to undetectable levels.

In certain samples from 12 of the 45 patients, PRP was detected in the form of a complex digestible with acid-pepsin. The complex was detected in concentrations of 1.5-60 ng/ml for periods ranging from 1 to 145 days after admission. Generally (10 of 12 patients), free antigen was detected in early samples, the complexed PRP appearing thereafter (Fig. 8). In each case, complex was detected at the time of development of detectable net antibody and thus presumably represents an immune complex.

*Relation of antigenemia to clinical status.* The initial concentration of PRP in CSF and serum correlated with the clinical status at the time of hospitalization and, to a lesser extent, with severity of hospital course. The geometric mean serum concentration in patients presenting with severe illness was 54 ng/ml, (range:

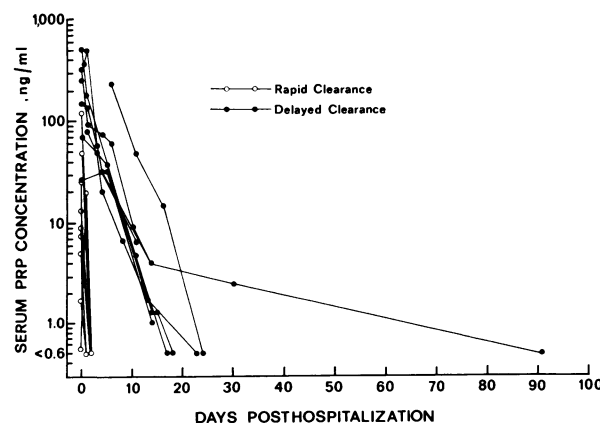


FIGURE 1 Kinetics of clearance of PRP from serum. The data represent the results of assays for PRP in sequential serum specimens from 33 patients samples with sufficient frequency to allow examination of clearance patterns and rates.

< 0.5-1,100 ng/ml) while that in patients with moderate or mild symptoms was 10 ng/ml (range: < 0.5-140 ng/ml) ( $P < 0.005$ ) (Fig. 2). The geometric mean of serum concentrations of those with severe hospital courses was 59 ng/ml (range 0.5-1,100) while that of the other patients was 13.5 ng/ml (range 0.5-500) ( $P < 0.05$ ) (Fig. 2). Seven of eight children with admission serum concentrations of < 40 ng/ml had relatively benign hospital courses.

As shown in Fig. 3, the duration of antigenemia in patients with a severe hospital course was prolonged (geometric mean of 7.9 days) when compared with that of patients with less severe courses (geometric mean of 2.0 days) ( $P < 0.0025$ ). Of 21 children who were antigen-free by hospital day 2, only 2 had severe courses. Duration of free antigenemia and the number of febrile hospital days, were also positively associated with a correlation coefficient of 0.39, ( $P < 0.005$ ). When the duration of antigenemia was redefined in terms of total PRP, i.e., free and complexed PRP, the correlation between antigenemia and clinical course was even more striking. Patients with severe clinical courses had a mean period of antigenemia of 16.5 days, while those with a relatively benign course had a mean period of 2.5 days ( $P < 0.001$ ).

16 of the 45 patients had complications of their disease or possible chronic foci of infection. Seven patients had proven subdural effusions, one of which was cul-

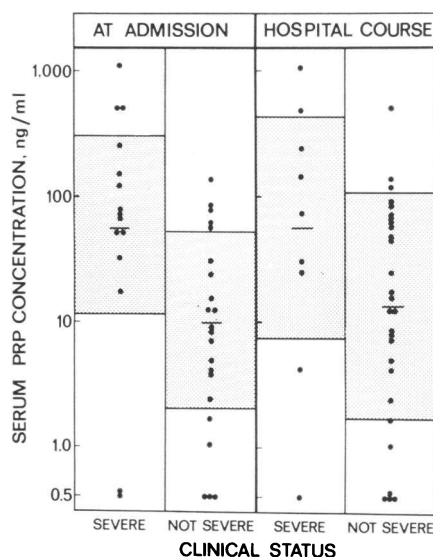


FIGURE 2 Comparison of the concentrations of PRP in serum samples obtained at the time of admission from patients with or without a severe clinical presentation or hospital course. Each closed circle represents the admission serum PRP concentration observed in one patient. Values plotted as horizontal bars within the stippled areas represent geometric means; the stippled areas represent  $\pm 1$  SEM.

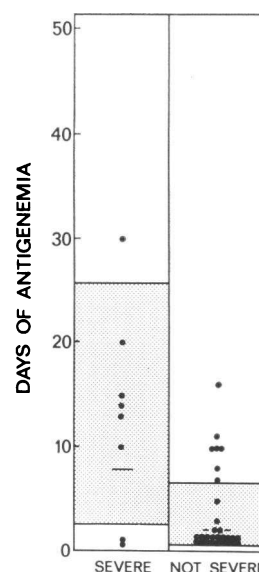


FIGURE 3 Comparison of the duration of detectable antigenemia observed in patients with or without severe neurological symptoms during hospitalization. Results are plotted as described in Fig. 2.

ture-positive 11 days after hospitalization; the other effusions were sterile. Small subdural effusions were suspected on the basis of brain scan or transillumination in an additional four cases; two patients had sinusitis diagnosed during hospitalization; lateral vein thrombosis was suspected but not proven in one case; isolated cases of cellulitis and of arthritis were also documented. The duration of free antigenemia in these patients (mean = 7.2 day) differed significantly from that (mean 1.8 days) of patients without complications ( $P < 0.001$ ) (Fig. 4). There was considerable overlap between these groups in terms of duration of antigenemia, but only 3 of the 23 children without detectable serum antigen on day 5, developed complications. All children with antigenemia after day 10 had documented complications.

**Antibody responses.** Of 38 admission sera studied, sufficient volume for antibody detection was available for 25. Of these, antibody was undetectable in 8, and in 10 others, levels were below the antibody concentrations of age-matched healthy children (Fig. 5A). These low initial antibody values could not be considered artifacts of inhibition by circulating antigen since PRP in complex form was detected in only two of the admission sera. However, the possibility that these low levels reflect consumption of preinfection antibody stores in the response to bacterial invasion cannot be discounted.

Seven patients had admission antibody levels exceeding the mean for age-matched controls. However, five of the seven had experienced symptoms of menin-

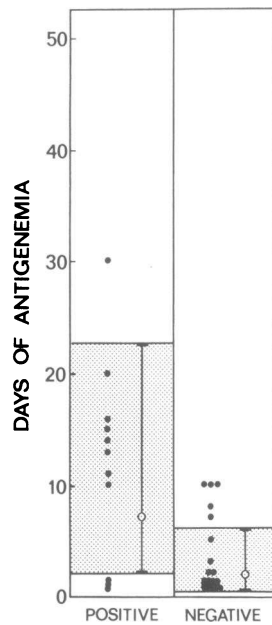


FIGURE 4 Comparison of the duration of detectable antigenemia observed in patients with or without focal effusions or infections complicating resolution of meningitis. Results are plotted as described in Fig. 2.

gitis including high fever and vomiting for 2 or more days before hospitalization, another had received 14 days of oral Ampicillin for otitis media. The serum concentration of PRP was undetectable or exceedingly low ( $<4$  ng/ml) in five of the seven, and was cleared in the 1st day of treatment in each case. In the one patient with a serum antibody level of 100 ng/ml, 86% of the PRP detectable in the serum was in the form of complex. It is thus likely that at the time of admission these patients were already clearing antigen from the circulation and mounting an immune response.

44 of the 45 patients were followed through maximal development of their immune response, or for at least 100 days after hospitalization. Some were evaluated for periods of up to 600 days. Fig. 5B presents the maximum antibody concentrations observed during the first 100 days of follow-up. Only 3 of the 44 patients had maximum antibody levels that were lower than the mean for age-matched healthy children. 6 of the 42 failed to show an increase in antibody concentration during this 100 day period. Four of these nonresponders subsequently developed low levels of antibody 200–400 days after hospitalization. Thus, an increase in antibody concentration was eventually seen in 42 of the 44

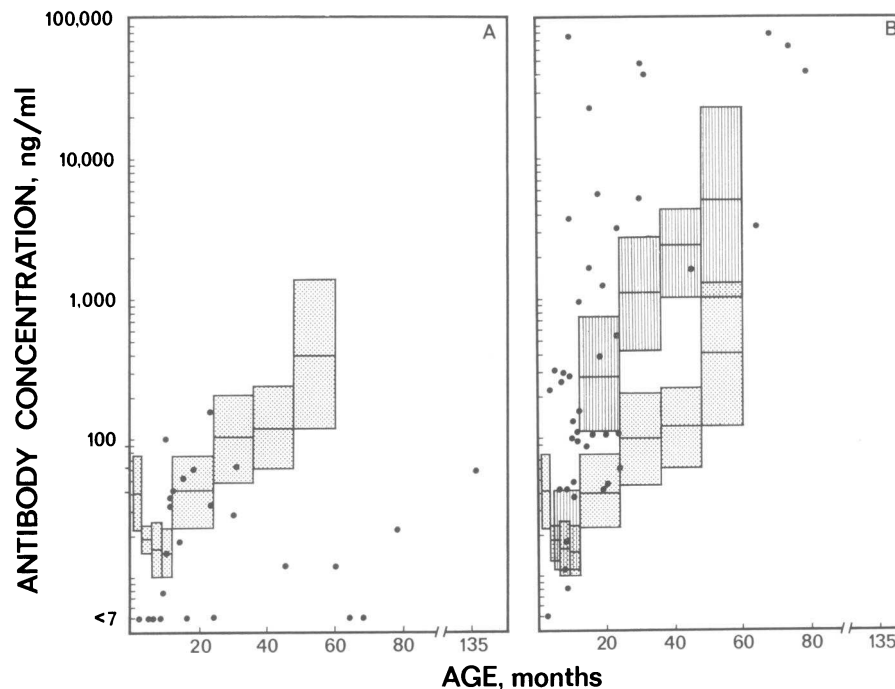


FIGURE 5 Relation of anti-PRP antibody activity to age of patient: (A) on admission and (B) maximal antibody response observed during 100 days after admission for therapy. Each closed circle represents the titer of an individual patient. The heavy bars within each stippled area in Figs. 5A and B represent the geometric mean titer for healthy children of the designated age; the stippled areas represent  $\pm t_{0.95}$  SEM. In Fig. 5B, the lined areas represent the antibody responses observed in 141 healthy children vaccinated with purified PRP, similarly plotted according to age groups. The limit of detection of antibody activity was  $<7$  ng/ml.

patients (95%), including 20 of 21 (95%) 1 yr or less in age.

The magnitude of the antibody response was directly correlated with the age of the patient (Fig. 5B) ( $r = 0.30$ ,  $P < 0.05$ ). However, as shown in Fig. 6, a more impressive inverse correlation was found between the duration of antigenemia, regardless of the age of the patient, and the magnitude of the immune response ( $r = -0.66$ ,  $P < 0.001$ ).

Analysis of circulating free and complexed antigen and antibody in sequential serum samples revealed two patterns of response. Fig. 7 presents an example of the response seen in 19 (52%) of the 36 patients serially examined. There was rapid clearance of PRP within the first 24-48 h followed by a prompt, and frequently rapid development of antibody to peak concentrations which exceeded 400 ng/ml (the mean anti-PRP antibody level in normal, presumably immune 5-yr-old children) in 16 of the children.

The patients presented in Fig. 8 and 9 are representative of a group comprising the other 17 patients sequentially evaluated, who failed to clear PRP within the first 24-48 h of hospitalization. Free PRP detectable in these patients for a mean period of 10.3 days. Six pa-

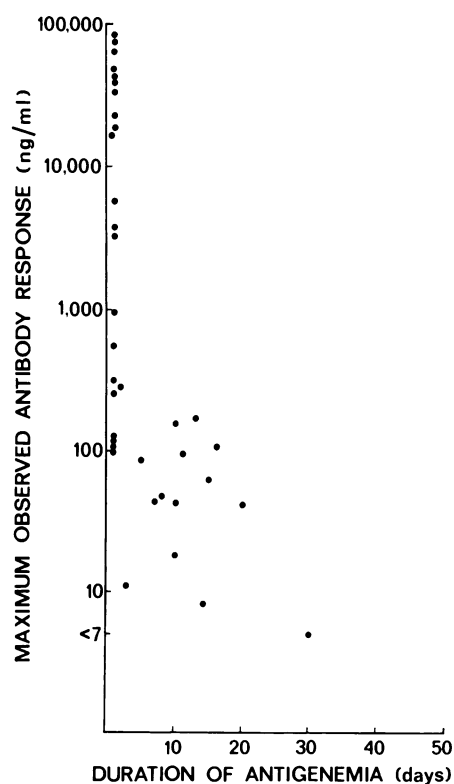


FIGURE 6 Correlation of maximum observed anti-PRP antibody activity and duration of antigenemia. Each closed circle represents the data of an individual patient. ( $r = -0.66$ ,  $P < 0.001$ ).

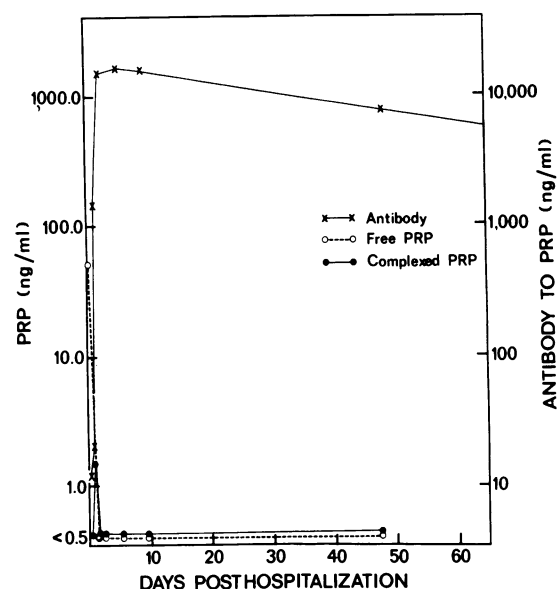


FIGURE 7 Clearance of circulating free and complexed PRP and development of anti-PRP antibody in a representative patient effecting rapid removal of antigen.

tients had PRP complex circulating after initial clearance of unbound PRP, the mean duration of antigenemia detected in either form thus being 22.8 days. Patients in this group were younger (median age 10 mo) than those affecting rapid clearance (median age 24 mo). Irrespective of age, the antibody responses recorded in this group were of low magnitude (e.g., Fig. 8) (geometric mean = 52 ng/ml) the highest antibody level detected in the first 100 days of convalescence being 310 ng anti-PRP/ml. Of the six children who failed to develop a detectable increase in antibody in response to infection, five were adequately studied for antigen clearance, and were each found to have had prolonged antigenemia. Two of these patients 100 and 150 days after hospitalization have yet to show any response (e.g. Fig. 9), and currently maintain levels of 23 ng/ml and less than 7 ng/ml anti-PRP antibody, respectively. Three patients developed low levels of anti-PRP activity 234, 284, and 431 days after hospitalization, indicating at least their capacity to respond to antigenic stimulation. The sixth patient who failed to respond to infection did develop antibody after immunization with a 25- $\mu$ g dose of PRP 50 days after hospitalization.

## DISCUSSION

Antigens of several genera of bacteria may be immunologically detected in body fluids during systemic infection. In 1971, Dochez and Avery were able to detect, by specific precipitation, polysaccharide antigens of *Pneumococcus* types I, II, or III in the serum of 26 and in the urine of 33 of 88 patients with pneumococcal

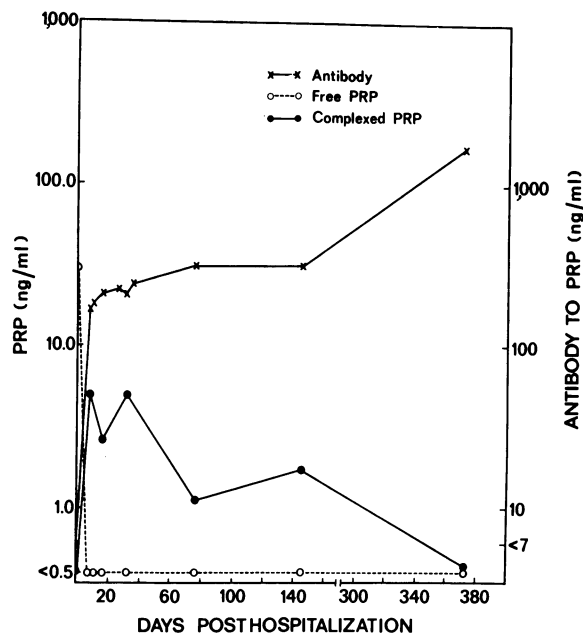


FIGURE 8 Clearance of circulating free and complexed PRP and development of anti-PRP antibody in a patient with prolonged antigenemia. Development of maximal antibody titers was delayed more than 145 days until clearance of PRP-complex.

pneumonia (19). The presence of detectable levels of antigen was associated with high mortality rates. Antigenuria in survivors was often prolonged, in one case, lasting 58 days. Alexander (20) and later Warburton et al. (8) extended these findings to children with *H. influenzae* b infection. Antigen concentrations were said to correlate with clinical status, and were used to assess the adequacy of antiserum therapy.

The introduction of immunologic methods more sensitive than precipitation has renewed interest in the clinical application of the detection of bacterial antigens. Counter-current immunoelectrophoresis (CIE) has now been employed to detect type-specific polysaccharides of meningococci (21, 22), pneumococci (23), streptococci (24), and *H. influenzae* b (10-12) in body fluids of individuals with systemic disease; agglutination of latex particles coated with antiserum has also been used to demonstrate PRP (9, 25). The radioimmunoassay, not heretofore used for clinical detection of bacterial polysaccharides, offers certain advantages in that it is quantitative and possesses a greater degree of sensitivity. The limits of sensitivity of this assay are 0.5 ng PRP/ml, while that of latex agglutination and CIE are 1-5, and 10 ng/ml, respectively<sup>3</sup> (4). Since 15% of the test specimens contained less than 2 ng/ml and 35% less than 10 ng/ml, the radioimmunoassay, although

<sup>3</sup> Manuscript in preparation.

technically more difficult, permitted a more thorough study of antigenemia.

The quantity of invading bacteria must be the most important determinant of initial PRP concentrations in serum or CSF. Thus, it might be expected that children with the highest concentration of antigen would have the most severe clinical presentations. Our observations support this presumption. The prognostic value of the PRP concentration in initial serum or CSF samples is less striking. Although PRP antigen concentrations in children with severe clinical courses are significantly greater than those with more benign courses, there is considerable overlap (Figs. 2 and 3). However, only 15% of children who had <6 ng/ml PRP serum on day 2 had severe symptoms during hospitalization and only 18% had a complicated course. Furthermore, all children with benign courses were found to be free of detectable antigenemia after the 10th day of hospitalization. Thus, follow-up quantitation of antigenemia may have prognostic value and should be a useful adjunct in the assessment of children with meningitis who have prolonged fever or a question of metastatic, or inadequately treated foci. The value of this parameter as a prognostic indicator of significant neurologic sequelae in children who recover from *H. influenzae* b meningitis is now being assessed.

The kinetics of the clearance of bacterial polysaccharides released during infection have not been described previously in man. In the nonimmune mouse, small intravenous doses of 100 µg of type III pneumo-

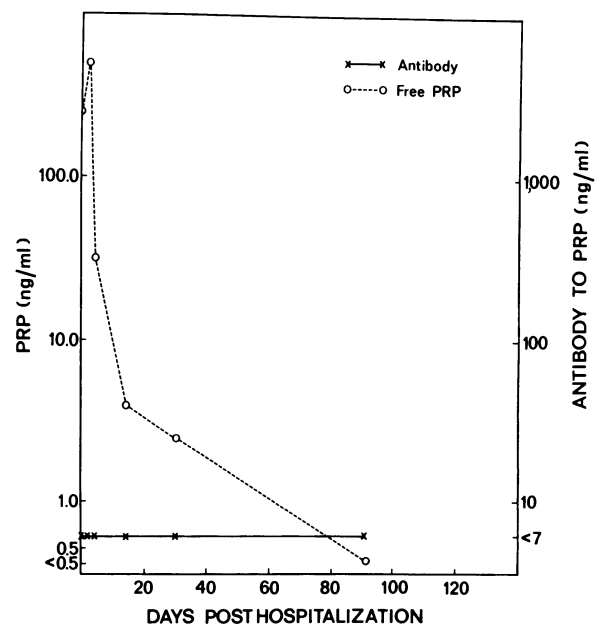


FIGURE 9 Clearance of circulating PRP in a patient with prolonged antigenemia. PRP-complex was not detected. To date, no antibody response has been detected.

coccal polysaccharide are rapidly cleared from the circulation within 24 h (26). In contrast, administration of "paralytic" doses of 1 mg of type II pneumococcal polysaccharide results in persistence resistance of low levels of polysaccharide in the circulation for periods in excess of 100 days (27).

Similar features of clearance were observed in our patients. About one-half of the children with *H. influenzae* b meningitis cleared free PRP from the serum within 24 h; the duration of antigenemia in the other children varied, and was often prolonged especially in patients with very high circulating levels of PRP on admission. The mechanism of clearance has not been defined. It is clearly related to the patient's age, older children being more effective in eliminating antigenemia. The lack of a significant correlation between age and initial serum and CSF antigen concentrations suggests that the prolonged antigenemia observed in younger children does not result from greater quantities of invading bacteria. Antibody is known to facilitate elimination of pneumococcal polysaccharide injected into mice (26), and probably played a role in the clearance of PRP from certain children, particularly the older ones. Other mechanisms must also be important, however, for the initial exponential phase of clearance began in patients before the appearance of antibody or complex, and PRP was cleared from the circulation of children who never produced detectable antibody. In this regard, the role of the reticuloendothelial system in PRP clearance deserves careful evaluation. Kaplan et al. (28) showed that pneumococcal polysaccharides are preferentially taken up by fixed macrophages, and may remain in the reticuloendothelial system for prolonged periods of time. Subsequent studies (29, 30) demonstrated that these antigens persist in tissues in an immunogenic form. Howard et al. concluded from their studies that recirculation of pneumococcal polysaccharide from such nonmetabolized tissue stores was the basis for the chronic low-grade antigenemia observed in mice given a large dose of polysaccharide (27). Whether these findings are applicable to the clearance of PRP in man will require further study. However, the kinetics of polysaccharide clearance observed in these animals are strikingly similar to those observed in our patients with sustained antigenemia. In view of the observed relationship between patient age and efficiency of clearance, the possibility of a developmental deficiency in macrophage function must be entertained.

In infections with *Escherichia coli*, the red cells have been found to adsorb bacterial antigens (31). PRP binds to washed red cells in saline (8); however, addition of nanogram quantities of [<sup>3</sup>H]PRP to normal human blood in vitro results in very little adsorption to red

cells.<sup>4</sup> Thus, in *H. influenzae* b infection, the red cells are probably an insignificant reservoir of antigen. Enzymatic degradative systems must also be considered. For example, pancreatic RNAase degrades PRP in vitro (32). Similar enzyme activity is found in human serum. The ultimate fate of the PRP has not been evaluated. Our experience with a few urine samples<sup>4</sup> and studies with pneumococcal antigens indicate that degraded polymer is excreted through the kidneys (33, 34).

Many of these children had symptoms of respiratory illness or meningitis for significant intervals before hospitalization. It is reasonable to assume that most of them had exposure to *H. influenzae* b before the collection of our initial serum specimens. Thus, antibody activity in those specimens might not have reflected the child's preinfection antibody status. The observation, therefore, that antibody activity was deficient or absent in most of the admission specimens supports the hypothesis that anti-PRP plays a role in natural immunity to *H. influenzae* b.

Significant increases in anti-PRP antibody concentration were detected in 38 of the 44 patients within 100 days of admission for treatment. Thus, most of the children, including those under 1 yr, were capable of immunologic response to infection. These results differ from those obtained when antibody activity was assayed by other methods (5-7). Differences reflect primarily the greater sensitivity of the radioimmunoassay. (6 ng anti-PRP antibody/ml.) Bactericidal and hemagglutination assays have detection limits of approximately 1,000 and 500 ng/ml of antibody, respectively (17). Our findings indicate that antibody responses are related directly to the patient's age and antigen load, and that free antibody is detected only after the clearance of PRP, which may take weeks. Since previous interpretations of the antibody responses of children with *H. influenzae* b disease have generally been based on specimens collected on admission and 2-3 wk later, they may be incomplete (4-7). A cautionary note regarding the potential need for prolonged follow-up of serum specimens thus seems warranted.

Although the antibody response of most of the children was statistically significant, the concentration of anti-PRP antibody required for immunity to *H. influenzae* b is not known. In infant rats, experimentally infected (35), protection by human anti-PRP antibody is demonstrable at concentrations as low as 40 ng/ml in the animals' serum. Estimates of the protective level in human infection are complicated by the possibility that antibodies to surface antigens other than PRP may play a role in naturally acquired immunity (36, 37). The importance of anti-PRP antibody, however, is

<sup>4</sup> Unpublished observations.



underscored by findings in two patients in the present study who developed a second systemic infection with *H. influenzae* b within a year after recovery from their first. After the initial infection, both had had delayed clearance of PRP and low peak anti-PRP responses (38 and 44 ng/ml). In both patients, the anti-PRP antibody had dropped below the detectable level by the onset of the second infection. The second bacterial isolates were serologically distinguishable from the first. At the time of their second admissions, both patients had non-PRP-absorbable bactericidal antibodies directed against the initial infecting strains. However, no bactericidal activity was detected against the strains producing the second infections.

6 children, including 4 of 21 children 1 yr or less in age, failed to develop antibody to PRP in response to infection. Similar states of prolonged unresponsiveness have been readily induced in very young animals by administration of large doses of antigen (38-42), the extreme and classical example being the state of immunologic paralysis to pneumococcal polysaccharide antigen described by Felton et al. (43). It remains to be determined if any of these children were immunologically paralyzed in the sense of permanent absence of response to PRP. Four of the children who failed to respond within 100 days after infection did develop antibody late in convalescence, one in response to secondary immunization with a PRP vaccine. The other two cases have cleared circulating antigen but are without antibody 100 and 150 days after hospitalization. Delayed antigen clearance was a prominent feature in each of the six cases. The kinetics of antigen clearance and antibody response of certain of the children with no or low initial humoral responses resemble those describing a state of pseudo-paralysis distinguished by Howard et al. (44), in which the absence of circulating antibody is due to trapping by circulating antigen followed by destruction of the trapped antibody and recycling of nonmetabolized antigen (27). Such "pseudo-paralysis" would have different implications from permanent paralysis for young patients with systemic *H. influenzae* b disease. For example, the possibility of overcoming the antibody-binding activity of antigen by passive immunization would deserve consideration. Since children who will produce low or no antibody activity can be predicted by the presence of circulating antigen and the absence of free antibody on days 10-14 of hospitalization, the population at risk could be identified with some certainty. The potential of inducing an immune complex disorder by passive immunization must be considered, but such risks must be balanced against the apparent high chance of secondary disease in such children.

Comparison of the antibody response to PRP immunization to that developed after infection is of consider-

able interest. When 141 children 5-59-mo old were given single intramuscular injections of 0.67, 3.3, 17, or 67  $\mu$ g of purified PRP, the frequency and magnitude of antibody increase was distinctly lower at 67  $\mu$ g than at the intermediate doses. This dose effect, however, was largely confined to the children below 2 yr of age (45). While the antigen dose rendered by infection cannot be determined from our data (owing to the uncertain dynamics of generation, degradation, and elimination), the duration of antigenemia might be taken as one reasonable estimate of dosage. Thus, in infection the higher "doses" were also generally accompanied by weaker antibody responses (Fig. 6). This finding, however, may bear only a superficial resemblance to the supraoptimal dose effect seen in vaccination, for the dose size in infection may not be an independent variable and may depend significantly upon the capacity for clearance (Fig. 1).

Maturation of the capacity for antibody responses with age varies for different classes of antigens but is found to a certain degree with all. An increasing response to PRP vaccination among infants, children, and adults was first reported by Robbins et al. (46). Our above-mentioned vaccination study corroborated this finding; further, the range of dose size employed permitted the age-response effect to be distinguished from the inhibitory effect of high doses upon younger children (45). The maximal titers postinfection in the present study and the mean postvaccination titers of 141 children are compared as a function of age, irrespective of dose, in Fig. 5B. Above the age of 1 yr, titers of the two groups are similar.

Below 1 yr a contrast is suggested. Of 23 infants 2-3-mo old vaccinated with 5- or 10- $\mu$ g doses by Robbins et al., 20 had at least slight, transient antibody increases (46), but in a second study, only 5 of 27 infants had detectable increases during the 10 mo after a single dose of 10  $\mu$ g.<sup>5</sup> In our initial study only 5 of 18 infants 5-12-mo-old had increases within 6 wk after injection (45); (data included in Fig. 5B). In our current vaccination study only 5 of 37 infants receiving 10  $\mu$ g and 0 of 12 receiving 1- $\mu$ g doses have had rises detectable at 2 or 4 mo postvaccination.<sup>3</sup> Thus, although there are certain differences between the vaccine preparations and radioimmunoassays employed in the several studies, the infants' response to the isolated polysaccharide appears inconsistent and scanty. In infection, however, 17 of 21 infants had detectable rises within 6 wk. The same assay was employed as in our vaccination studies.

This difference resembles a phenomenon well established in experimental animals and in man: isolated bacterial capsular polysaccharides are poor primary im-

<sup>5</sup> Robbins, J. B., personal communication.

munogens, while the corresponding intact bacteria readily induce antipolysaccharide antibody (47, 48).

The mechanism for this amplification of response is likely to include features of the carrier-hapten effect, in which the hapten-specific antibody response of bone marrow-derived lymphocytes is stimulated or amplified through the cooperation of thymus-derived lymphocytes sensitized to a carrier molecule (49). Thus, the primary response to purified type III pneumococcal polysaccharide may be accentuated by immunization with polysaccharide coated on sheep red cells if the recipient animals have received prior immunization with the sheep red cell carrier (50). Prior sensitization of the host with an unrelated bacterium such as *Corynebacterium parvum* may also augment the antibody response to capsular polysaccharide (51), a phenomenon possibly related to the immunopotentiating effect of macrophages activated by intact bacteria. With gram-negative bacteria, the potential adjuvant action of cell wall lipopolysaccharides (52, 53) might also be involved.

The maximal incidence of *H. influenzae* b meningitis occurs in the 1st yr of life, and thus the optimal vaccination program would have to induce immunity in this age range. The present study suggests that, despite the apparent poor immunogenicity of purified PRP in infants, the antigen presented in more complex form might be effective. Support is thereby given to the hypothesis that enteric bacteria possessing antigens cross-reactive with PRP are a source of natural immunization against *H. influenzae* b and potentially a means of vaccination (54). Strongly encouraged also are efforts to sort out the contribution of the various components of *H. influenzae* b to the primary immunogenicity of its capsular polysaccharide.

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