Enzyme-Linked Immunosorbent Assay for Detection of Capsular Antibodies Against *Haemophilus influenzae* Type b: Comparison with Radioimmunoassay

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An enzyme-linked immunosorbent assay (ELISA) is described for detection of anti-capsular antibodies against *Haemophilus influenzae* type b. Polyribosephosphate was covalently bonded to poly-L-lysine before adsorption to microtiter plates. ELISA immunoglobulin G and immunoglobulin M anti-polyribosephosphate antibody titers were comparable to total anti-polyribosephosphate antibody concentration determined by radioimmunoassay. The ELISA technique will be useful for further investigations of host response to infections due to *H. influenzae* type b but is not intended to be used as a serological method for documenting *H. influenzae* type b infections.

The enzyme-linked immunosorbent assay (ELISA) technique has been used to determine concentrations of antibody directed against the capsular polysaccharides of *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (1, 3, 4, 9, 11, 13). ELISA offers a rapid, relatively inexpensive method to quantitate and differentiate immunoglobulins to capsular polysaccharide. We describe an ELISA method for detecting antibody directed against the polyribosephosphate (PRP) capsule of *H. influenzae* type b and compare ELISA results with those obtained by radioimmunoassay (RIA). This ELISA method can be established in most laboratories and may be potentially important should an effective vaccine directed against the PRP of *H. influenzae* type b become available for young children.

MATERIALS AND METHODS

Preparation of PRP-coated plates. PRP alone did not adsorb consistently to microtiter plates. Prior coating with burro anti-PRP antibody (kindly provided by John B. Robbins, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.) resulted in high non-specific adherence of test sera. Thus, *H. influenzae* type b capsular polysaccharide (kindly provided by Robert S. Baker, Lilly Research Laboratories, Indianapolis, Ind.) was coupled to poly-L-lysine by the method of Gray (6). Briefly, 0.1 ml (1,000 µg/ml) of a solution of PRP in deionized water was added to 0.5 ml of 0.01 N NaOH with 0.001% phenolphthalein indicator. After alkalization of the PRP, the solution was added to 0.5 mg of cyanuric chloride (Sigma Chemical

Co., St. Louis, Mo.) and mixed for 10 s. This mixture was added to 0.1 ml of 0.1% poly-L-lysine (type VII-B; Sigma Chemical Co.), mixed, and refrigerated at 4°C for 2 h. A control poly-L-lysine solution was prepared in an identical fashion except deionized water without PRP was used. After 2 h, the PRP solution was diluted in Dulbecco phosphate-buffered saline (PBS) without calcium or magnesium (GIBCO Laboratories, Grand Island, N.Y.) to obtain a PRP concentration of 1 µg/ml. The control poly-L-lysine solution was diluted similarly. Alternate columns of round-bottomed microtiter well substrate plates (Immulon AUS-coated; Dynatech Laboratories, Inc., Alexandria, Va.) were filled with 200 µl of either PRP-poly-L-lysine or control poly-L-lysine per well. The plates were stored at 4°C without washing until used. Plates could be stored in this manner for at least 3 months, and there was excellent reproducibility of serum titers (within one to two dilutions) from batch to batch of PRP-poly-L-lysine-coated plates.

ELISA procedure. The ELISA procedure was modified from that of Barrett et al. (1). Plates were washed three times with 200 µl of PBS, pH 7.4, containing 0.05% polysorbate (Tween 20) per well before use in the assay. Twofold serial dilutions (lowest dilution 1:4 or 1:8) of human sera were prepared in PBS (containing 0.05% Tween 20 and 1% bovine serum albumin; Sigma Chemical Co.), and 100-µl volumes were added in triplicate to PRP-poly-L-lysine- and poly-L-lysine control-coated wells. Optimal time of incubation at 4°C was 4 h. After incubation, the plates were washed three times, and 100 µl of goat enzyme-conjugated anti-human immunoglobulin (anti-human immunoglobulin G [IgG] [γ chain specific] alkaline phosphatase conjugate or anti-human IgM [μ chain specific] alkaline phosphatase conjugate; Sigma Chemical Co.) was added to each well. The optimal dilution of the anti-

human immunoglobulins was 1:1,000 in PBS, as determined by checkerboard titration. The plates were incubated for 2 h at 37°C and washed three times. A 200- μ l amount of *p*-nitrophenyl phosphate substrate (1.0 mg/ml) in diethanolamine buffer (pH 9.6) was added to each well, and the plates were incubated at room temperature for 60 to 90 min. Optical densities were determined at 405 nm with an MR530 Micro-ELISA Reader (Dynatech Laboratories, Inc.). A result was considered positive if all three of the triplicate samples in the PRP-poly-L-lysine-coated wells exceeded the mean plus three standard deviations of the triplicate samples of the same dilution in the corresponding poly-L-lysine control-coated wells (2, 10).

Anti-PRP antibodies were determined by RIA in the laboratory of J.C.P. (12). A 125 I-labeled derivative of *H. influenzae* type b polysaccharide was prepared by a modification of the method of Gotschlich et al. (5). *H. influenzae* polysaccharide (5 mg/ml) was brought to pH 10.5 with 0.1 M sodium hydroxide. Cyanogen bromide (100 mg/ml) at 0.3 mg/mg of polysaccharide was added, and the pH was maintained at 10.5 with 0.1 M sodium hydroxide for 6 min at 4°C. Tyramine (50 mg/ml) and 0.5 M sodium bicarbonate in equal weight to the polysaccharide were added. (The pH of the mixture was 8.5.) The mixture was tumbled at 4°C for 16 h, centrifuged, and passed through a Sephadex G-25 column in PBS. This product was radiolabeled with 2 mCi of 125 I by the chloramine-T method (8).

An adult serum (SK, immunized with 50 μ g of the purified *H. influenzae* type b polysaccharide) containing 40 μ g of antibody per ml (as determined by quantitative precipitation) served as the reference standard. Pooled plasma from three unimmunized adults served as an internal control. Fetal calf serum, assayed for the absence of immunoglobulins, was used to dilute the reference serum and other sera with high antibody levels. A 50- μ l serum sample and 10 μ l of 125 I-labeled (0.1 μ g/ml) antigen were delivered to a microfuge tube, mixed, and incubated at 4°C overnight. A 60- μ l amount of saturated ammonium sulfate was added, mixed, and after 1 h at room temperature, centrifuged for 5 min. A 50- μ l sample of the supernatant was removed and counted for 5 min in a gamma-counter. Antibody levels, expressed as micrograms per milliliter, were determined in comparison to the SK standard curve.

The sum of the log transformation of IgG and IgM titers was compared to the total anti-PRP antibody content by RIA by linear regression analysis. For purposes of analysis, all nondetectable ELISA titers and anti-PRP antibody concentrations of <0.025 μ g/ml were assigned a value of zero.

Serum samples. Sera were obtained from 29 children hospitalized with infection due to *H. influenzae* type b (meningitis, 24; epiglottitis, 4; cellulitis, 1). Acute and convalescent sera from the same patient were run on the same microtiter plates. The volumes of some samples limited the lowest dilution to $\geq$ 1:16. Samples of sera were stored at -70°C. Pooled normal human serum was prepared from the sera of healthy adult volunteers.

Pooled normal human serum was adsorbed with an overnight growth of *H. influenzae* type b, *H. influenzae* nontypeable, or *S. pneumoniae* for 18 h at 4°C. Bacteria were removed by high-speed centrifugation and membrane filtration.

2-Mercaptoethanol (2-ME) (0.2 M) was added in equal volumes to sera from six healthy adult volunteers and incubated at 37°C for 1 h to inactivate IgM (1). Log transformations of antibody titers for 2-ME-treated sera and untreated sera incubated in saline were compared by paired *t*-test.

RESULTS

Adsorption studies. Pooled normal human serum had an ELISA IgG titer equal to 1:2,048 and an IgM titer of 1:128. When adsorbed with nontypeable *H. influenzae* or *S. pneumoniae*, the IgG and IgM ELISA titers were unchanged. However, after adsorption with *H. influenzae* type b, the IgG and IgM ELISA titers were both 1:32.

2-ME inactivation. The ELISA IgG and IgM anti-PRP antibody titers for sera from six healthy adults before and after 2-ME inactivation are shown in Table 1. There were no differences in the IgG titers, but the IgM titers were significantly reduced ($t = 8.37$, $P < 0.001$) after 2-ME inactivation of sera.

ELISA and RIA anti-PRP antibody determinations. The IgG and IgM anti-PRP antibody titers by ELISA are compared with anti-PRP antibody concentrations by RIA in sera from 29 children with *H. influenzae* type b infections in Table 2. Children with nondetectable anti-PRP antibody by RIA generally had no detectable titers (<1:4 or <1:8) by ELISA also (except for three patients). The sum of the log IgG and log IgM titers correlated significantly ($r = 0.640$, $P < 0.001$) with the total anti-PRP antibody content by RIA. Early and late serum samples were available in 13 children. In all instances, there was complete agreement between ELISA and RIA in determining whether an antibody response had occurred. Patients 17 and 24 were of particular interest. Both children initially had anti-PRP antibodies that were predominately of the IgM class but that on follow-up were predominately of the IgG class.

TABLE 1. IgG and IgM anti-PRP antibody titers before and after treatment of sera with 2-ME

Sample no.	IgG titer		IgM titer	
	PBS ^a	2-ME ^b	PBS	2-ME
1	1:1,024	1:1,024	1:32	<1:8
2	1:256	1:256	1:64	<1:8
3	1:2,048	1:2,048	1:512	<1:8
4	1:512	1:128	1:64	<1:8
5	<1:8	<1:8	1:128	<1:8
6	1:512	1:512	1:64	<1:4

^a PBS, Phosphate-buffered saline.

^b 2-ME, 2-Mercaptoethanol.

TABLE 2. Comparison of anti-PRP antibody content determined by ELISA and RIA

Patient no.	Age at admission (mo)	Days after admission	Anti-PRP ELISA titers		Anti-PRP antibody concn by RIA ($\mu\text{g/ml}$)
			IgG	IgM	
1	5	1	<1:4	<1:8	<0.025
2	5	1	<1:8	<1:8	<0.025
		12	<1:8	<1:8	<0.025
3	7	1	<1:4	<1:8	0.028
4	7	48	<1:8	<1:8	0.18
5	8	11	<1:8	<1:8	<0.025
6	9	1	<1:4	<1:8	<0.025
7	9	1	<1:8	<1:8	0.025
		205	<1:8	1:64	0.64
8	9	2	<1:4	<1:8	0.045
		13	1:128	1:64	0.92
9	10	1	<1:16	<1:25	0.11
10	10	1	<1:8	<1:8	<0.025
11	11	12	<1:4	<1:8	0.06
12	11	1	<1:8	<1:8	<0.025
13	12	1	<1:8	<1:8	<0.025
		12	<1:8	<1:8	<0.025
14	12	33	<1:8	<1:8	0.033
15	12	16	<1:8	<1:8	0.04
16	13	13	1:8	<1:8	0.42
17	14	1	1:16	1:32	0.34
		11	1:16	<1:4	0.18
18	15	455	<1:8	ND ^a	0.15
19	17	1	<1:4	<1:8	<0.025
		15	1:16	ND	0.37
20	26	1	<1:8	<1:8	<0.025
		33	<1:8	<1:8	<0.025
21	26	1	1:64	1:64	0.086
		12	1:2,048	1:64	27.0
22	30	57	1:1,024	1:256	3.6
23	30 ^b	1	<1:4	1:8	<0.025
		5	<1:4	1:256	2.7
24	35	5	<1:32	1:128	2.9
		53	1:128	1:32	3.1
25	39 ^c	1	1:16	1:64	<0.025
		6	1:128	1:512	26.0
26	39	1	1:16	1:64	1.6
		10	1:64	1:512	7.4
27	40 ^c	1	<1:8	<1:8	<0.025
		9	1:1,024	1:128	18.0
28	43 ^c	1	<1:4	1:4	0.025
		8	1:128	1:128	11.0
29	48 ^c	1	1:256	1:32	0.38
		64	1:2,048	1:128	3.3

^a ND, Not done.^b Cellulitis.^c Epiglottitis.

DISCUSSION

Neutral bacterial capsular polysaccharides do not adsorb reliably to polystyrene, and we found this to be the case for the PRP capsule of *H. influenzae* type b (7). Prior adsorption of burro anti-PRP antibody to polystyrene microtiter plates to capture PRP antigen resulted in excessive nonspecific adsorption of test sera. By using the simple method of Gray to covalently bind PRP to poly-L-lysine, we found the PRP-

poly-L-lysine preparation adsorbed reliably to polystyrene microtiter plates (6). The reproducibility of the titers for pooled normal human serum was within 1 to 2 dilutions, using different preparations of PRP-poly-L-lysine and different batches of plates.

Dahlberg described an ELISA method with capsular polysaccharide prepared from *H. influenzae* type b RAB (3). With this assay of 23 normal children between 1 month and 2 years of age, 19 had IgG and 17 had IgM antibody titers

to *H. influenzae* type b capsular polysaccharide. All seven children with *H. influenzae* type b meningitis (6 of 7 were ≥ 2.5 years of age), including one 6-month-old child, had anti-PRP antibody present on admission. There was no correlation between the antibody quantitation by ELISA which measured antibodies to capsular polysaccharide and bactericidal antibodies, which are antibodies to a variety of capsular and noncapsular antigens. Dahlberg did not compare anti-PRP antibody determinations by ELISA with RIA.

With the present ELISA technique, IgG and IgM anti-PRP antibodies generally were not detected in sera containing <0.025 μg of anti-PRP antibody per ml by RIA. Nine sera containing between 0.025 and 0.18 μg of anti-PRP antibody per ml by RIA did not have detectable ELISA titers at the dilutions tested. With these sera, if lower dilutions had been tested, antibody may have been detected by ELISA. Other sera containing ≥ 0.1 μg of anti-PRP antibody per ml by RIA had detectable ELISA titers of either IgG, IgM, or both classes. The ELISA titers correlated significantly ($P < 0.001$) with RIA determinations of anti-PRP antibodies by the method of analysis used. In general, the greater the anti-PRP antibody content by RIA, the greater was the combined IgG and IgM titer by ELISA. The discrepancies of IgG and IgM ELISA titers in patients with similar anti-PRP antibody concentrations may, in part, be explained by differences in antibody avidity. From this preliminary study, ELISA in our laboratory may not be as sensitive as RIA for determinations of anti-PRP antibody concentrations. However, the ELISA technique is less difficult and less expensive to perform than the RIA procedure and does not require a radiolabeled antigen, which is not easily prepared. Furthermore, a small number of specimens can be examined efficiently if so desired.

The ELISA technique can detect anti-PRP antibody and distinguish between IgG and IgM responses to PRP antigen. Anti-PRP antibody content of secretory IgA and IgG in sera has been measured by ELISA (4, 11). The ELISA technique will be useful in further studies of the host response to infections due to *H. influenzae* type b. This technique is not intended to be used as a serological method for documenting *H. influenzae* type b infections.

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