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A Rapid and Sensitive Chemiluminescence Assay for Evaluation of Functional Opsonic Activity of *Haemophilus influenzae* Type b-Specific Antibodies

EMMANUEL A. OJO-AMAIZE,[1]* JOSEPH A. CHURCH,[2] NOORI E. BARKA,[1] MELKON S. AGOPIAN,[1] AND JAMES B. PETER[1]

Specialty Laboratories, Inc., Santa Monica, California 90404-3900,[1] and Children's Hospital Los Angeles, Los Angeles, California 90027[2]

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Luminol-enhanced chemiluminescence (CL) of heterologous neutrophils was used to assess the capacity of a 1-ng/ml concentration of *Haemophilus influenzae* type b (Hib)-specific antibodies to induce opsonization of Hib with autologous heat-inactivated sera from children immunized with Hib capsular polysaccharide-polyribosylribitolphosphate (Hib-PRP) conjugate vaccine. Serum samples from 15 of 36 children (42%) vaccinated with Hib-PRP conjugate vaccine had protective levels of Hib-specific antibodies of ≥1,000 ng/ml. Ten of these 15 (67%) had poor or nonfunctional opsonic activity. Of the 10 children whose sera lacked opsonic activity, 5 (50%) presented with recurrent Hib infection. In contrast, none of the sera of 20 healthy adults lacked opsonic capability. CL intensity was proportional to the concentration of anti-Hib antibodies used for opsonization. Furthermore, the titers of Hib-PRP-specific antibody in children and adults did not correlate with opsonic activity. These results suggest that luminol-enhanced CL as described here with minute concentrations of antibody for opsonization can be used to assess functional capacity of anti-Hib antibodies after vaccination or natural infection in the evaluation of patients with recurrent infections.

Haemophilus influenzae type b (Hib), a leading worldwide cause of morbidity and mortality among young children (14), causes meningitis, bacteremia, septic arthritis, pneumonia, cellulitis, and epiglottitis. In most instances, antibody to Hib-polyribosylribitolphosphate (Hib-PRP) possesses biologic activities against the organism (14) and is effective in both the treatment (2) and prevention of disease (3). Accordingly, the measurement of specific Hib-PRP antibody responses has become a standard component of the diagnostic evaluation of immune functions in children with recurrent infections (5, 13). Of particular concern, however, are those children who appear to have normal antibody responses and yet present with recurrent Hib disease (8).

Here we report the use of luminol-dependent chemiluminescence (CL) by heterologous neutrophils to measure antibody function as reflected in opsonization capacity. The results demonstrate that although most children immunized with Hib-PRP conjugate vaccine responded by generating high concentrations of specific antibody, many fail to produce antibodies with opsonizing activity. This suggests that measurement of anti-Hib-specific antibody concentration alone may not be adequate for evaluating protective anti-Hib humoral responses in children.

MATERIALS AND METHODS

Bacteria. Encapsulated Hib (ATCC 9795 from American Type Culture Collection), representative of invasive Hib disease, was cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) in 5% CO_2 at 37°C. Bacteria were harvested in the log phase, autoclaved at 121°C for 30 min, and washed three times in Hank's balanced salt solution (HBSS; pH 7.4; Irvine Scientific, Irvine, Calif.). The washed bacteria were lyophilized and stored at 2 to 8°C until used for assay. The heat-killed bacteria retained their antigenicity as judged by the enzyme-linked immunosorbent assay (ELISA) described below.

Subjects. Serum specimens from 36 children (15 boys and 21 girls), ranging from 1 to 7 years of age, who were seen for either routine immunologic evaluation for primary or acquired immune deficiencies, recurrent Hib infection, otitis media, or sinusitis, were studied. Twenty healthy adult laboratory workers served as normal subjects.

Vaccines and vaccination schedule. Sera from all children were obtained after at least three immunizations with Hib-PRP conjugate vaccine (Hib Titer; Praxis Biologics, Inc., Rochester, N.Y.).

Antibody determination. Sera from children and adults were tested for immunoglobulin G (IgG) antibody against Hib-PRP by an ELISA as described by Barka et al. (4). Briefly, enzyme immunoassay plates were coated with Hib-PRP provided as a courtesy by Lederle Laboratories (Pearl River, N.Y.) coupled to tyramine. One-hundred-microliter volumes of diluted serum samples and controls were added to duplicate wells of the plates. Seven twofold serial dilutions of Hib standard (calibrated against the Food and Drug Administration Center for Biological Evaluation and Review standard; Carl Frasch, Bethesda, Md.) were also included in duplicate wells. After 30 min of incubation at room temperature, plates were washed three times with phosphate-buffered saline–Tween and blot dried. Appropriately diluted alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) was added, and the plates were incubated and washed as described above. The enzymatic reaction was developed with *p*-nitrophenyl phosphate disodium substrate (Sigma Chemical Co., St. Louis, Mo.). Spectrophotometric measurement was made at 405 nm with an ELISA microplate reader (Dynatech, Chantilly, Va.). Results were expressed in nanograms of antibody protein per milliliter from the standard curve.

IgG subclass determination. IgG subclasses (IgG1, IgG2, IgG3, and IgG4) were measured by immunoradiometric assay with monoclonal antibodies as described previously (1). The sum of the four subclasses measured by immunoradiometric assay was compared with the total IgG determined by nephelometry.

Opsonization. Following heat inactivation of sera at 56°C (water bath) for 30 min, each serum sample was diluted to 1.0 ng of Hib-specific antibody protein per ml. The desired quantity of Hib to be used in the assay was sonicated with a model 50 sonic dismembrator (Fisher Scientific, Tustin, Calif.) at a frequency of 18 kHz for 30 to 60 s. One hundred microliters of diluted serum was added to 1.5 mg of lyophilized, sonicated Hib in 100 μl of HBSS (phenol red free). The mixture was incubated for 30 min at 37°C (water bath) with shaking. After incubation, the mixture was centrifuged at 13,000 rpm for 5 min in a microcentrifuge. The supernatant was discarded; the pellet was washed twice with 200 μl of HBSS and centrifuged as described above, and the supernatant was removed. The pellet was resuspended in a final working volume of 100 μl of HBSS.

Neutrophil isolation from whole blood. For each experiment, 20 to 40 ml of heterologous whole blood was drawn in heparinized tubes from a healthy adult volunteer. Two milliliters of 6% dextran (Pharmacia, Uppsala, Sweden) solution

* Corresponding author. Mailing address: Specialty Laboratories, Inc., 2211 Michigan Ave., Santa Monica, CA 90404-3900. Phone: (310) 828-6543. Fax: (310) 828-6634.

TABLE 1. Controls for the CL assay

Type of treatment[a]	cpm, 10^6
PMNs + luminol + Hib opsonized with undiluted human serum	3.20
PMNs + luminol	3.04
PMNs + luminol + nonopsonized Hib	0.84
PMNs + HBSS	0.12
Nonopsonized Hib + HBSS	0.16
Nonopsonized Hib + HBSS + luminol	0.80
PMNs + nonopsonized Hib	0.09
HBSS + luminol	0.09

[a] PMNs, polymorphonuclear leukocytes.

was added to each 10 ml of whole blood and incubated at room temperature for 40 min to sediment the erythrocytes. At the end of the incubation, the plasma-rich layer was collected, and the erythrocyte layer was discarded.

The plasma- and leukocyte-rich layer was diluted 1:3 with HBSS, and 10 ml of the diluted fraction was layered onto 3 ml of Ficoll-Paque solution (Pharmacia) in 15-ml Falcon centrifuge tubes. The tubes were centrifuged in a Beckman model TJ-6 centrifuge at 2,000 rpm at room temperature for 20 min. Following centrifugation, the supernatant containing lymphocytes was discarded, leaving behind the neutrophil-rich pellet. The pellet was washed twice in phenol red-free HBSS. The cell concentration was adjusted to 10^6 cells per ml after checking viability by the trypan blue dye exclusion method.

CL. CL was determined essentially as described previously (6, 12) with some modifications. Briefly, 100 μl of a 15-mg/ml concentration of the Hib suspension (final concentration, 1.5 mg), preopsonized as described above, was added to 2 ml of the neutrophil suspension (10^6 cells per ml). Ten microliters of luminol (final concentration, 125 μM) was added to the reaction mixture in 10-ml dark-adapted plastic scintillation vials. The mixture was mixed gently by shaking and loaded onto a Beckman liquid scintillation counter (model LS3801) fitted with a single-photon monitor. The counter was run at ambient temperature and in the out-of-coincidence mode. Each sample was counted repeatedly over a 20-min period with intervals of 2 min. Control vials used as background or blanks contained essentially unopsonized bacteria, i.e., bacteria incubated at 37°C in 100 μl of HBSS. Pooled human AB serum with a high opsonic index (OI) was used as a highly positive control (see below). Bacteria were opsonized with undiluted pooled human AB serum. Neutrophils plus luminol alone could be used alternatively as a highly positive control.

CL was recorded in counts per minute (cpm). Mean cpm from all measurements obtained over a 20-min period were determined. Results were expressed as an OI derived from the following formula:

$$\text{OI} = \frac{\text{mean cpm of patient serum} - \text{mean cpm of blank}}{\text{mean cpm of highly positive control} - \text{mean cpm of blank}} \times 100$$

where patient represents bacteria opsonized with patient serum, blank represents unopsonized bacteria (incubated in HBSS), and highly positive control represents bacteria opsonized with undiluted pooled human AB serum or neutrophils plus luminol. All of the possible controls are listed in Table 1.

On the basis of the OI values obtained for the 20 serum samples from healthy adults and the 36 from children, an opsonic activity classification or reference range was established: OI of <10, nonfunctional; OI of 10 to 24, poorly functional; OI of 25 to 50, moderately functional; OI of >50, highly functional.

Statistical analysis. Data were analyzed by Student's *t* test (continuous variables).

RESULTS

Establishment of controls for the CL assay. To control for several variables in the CL assay, different combinations of reactants were assayed in the reaction mixture as shown in Table 1. As can be seen, the highest response was obtained with neutrophils incubated with bacteria which had been opsonized with undiluted human AB serum. The second highest response was obtained with luminol and neutrophils only. Apparently, luminol on its own seemed to be stimulating the neutrophils. Therefore, either of these two highly positive controls was used in the OI formula in Materials and Methods. Table 2 shows that the use of either of these highly positive controls for determining the OI of five different serum samples resulted in similar OI values for each serum. Although all other combinations shown in Table 1 could serve as negative controls (blank), it was deemed more appropriate to use neutrophils plus luminol plus nonopsonized bacteria as a blank to control for the opsonizing effect of all of the sera tested.

TABLE 2. Comparison of OI values of five serum samples obtained by use of two different highly positive controls in the calculation

Serum sample no.	OI value calculated with cpm obtained from:	
	Undiluted highly positive serum	PMNs + luminol only
1	103	97
2	82	77
3	52	49
4	87	82
5	123	115
$\bar{x} \pm$ SD	89.4 ± 26	84.0 ± 25

Establishment of inter- and intraassay CVs for the CL assay. To establish the run-to-run variability of the CL assay (interassay variation), 20 different runs of one serum sample were performed on 20 different occasions. The results, shown in Table 3, demonstrate that the interassay coefficient of variation (CV) was 14.4. For determination of the intraassay variation, 20 replicates of one serum sample (10 duplicates) were run on the same day in one assay. The intraassay CV was 7.9 (Table 3). These CVs are considered to be reliable, considering the fact that CL assays are known to be much more variable than assays that measure cytotoxic and cytopathic effects.

Establishment of inter- and intraassay CVs for the anti-PRP assay. Inter- and intraassay variability of the anti-PRP ELISA was established by testing three samples 10 times each in the same run (intraassay) and by testing three samples in duplicate in 10 different runs (interassay). The results are shown in Tables 4 and 5.

Level of opsonophagocytosis is dependent on dose of anti-Hib-specific antibodies. The concentration of anti-Hib-specific antibodies required to induce the maximal level of opsonophagocytosis was determined by using a patient serum sample with a high anti-Hib titer for opsonization at different dilutions. Figure 1 shows a direct relationship between the level of opsonophagocytosis and the concentration of anti-Hib antibodies used for opsonization. A moderate but significant opsonophagocytic activity was induced at a concentration of 0.1 to 10 ng of anti-Hib-specific antibody per ml. Because significant differences were not observed at these lower concentrations, all subsequent samples were diluted to 1 ng/ml and used for opsonization in subsequent experiments.

Demonstration that Hib bacteria opsonized with anti-Hib-specific antibodies induce CL in neutrophils phagocytizing the opsonized Hib bacteria. Figure 2 depicts an experiment in which two different serum samples were used in opsonization of Hib to enhance phagocytosis by neutrophils as judged by CL

TABLE 3. Establishment of inter- and intraassay CVs for the CL assay

Variability determined	Type of treatment	Mean OI	SD	CV (%)
Interassay	20 different runs of the same sample performed on different days	95	13.7	14.4
Intraassay	10 duplicates of the same sample run on the same day	102	8.1	7.9

TABLE 4. Establishment of intraassay CVs for the anti-PRP assay with OD values[a]

Sample no.	OD value		CV (%)
	Mean	SD	
1	1.025	0.058	5.7
2	0.418	0.025	5.9
3	0.076	0.005	6.1

[a] Intraassay variability was represented by three samples tested 10 times each in the same run.

intensity. As shown in Fig. 2, there was a significant difference ($P < 0.0001$) between the two serum samples in their capacity to induce opsonophagocytosis, even though they had the same anti-Hib antibody titer (1:900). As expected, Hib bacteria incubated in HBSS induced the lowest CL intensity, which decayed very quickly (Fig. 2). CL was measured over a 20-min period because phagocytosis is known to be completed within 20 min.

Functional capacity of Hib-PRP-specific antibodies in healthy adults. Results depicted in Table 6 demonstrate that each of the 20 normal, healthy adults tested had antibodies to PRP, the capsular polysaccharide of Hib. All of the sera had opsonic activity. Although all 20 adult serum samples had Hib-PRP antibody concentrations above the minimum protective level for unvaccinated individuals (i.e., 200 ng of antibody protein per ml) (11), the concentration of specific antibody in each serum sample did not correlate with opsonic activity. For example, serum samples 1 and 5 had almost identical concentrations of specific antibody (663 and 660 ng of antibody protein per ml, respectively). Serum sample 1, however, had a poor opsonic activity, with an OI of 12, whereas serum sample 5 had a high opsonic activity, with an OI of 83 (Table 6). Similarly, two serum samples (numbers 2 and 8), which had the same OI of 64, had anti-Hib antibody concentrations that differed from each other by 14-fold (1,306 versus 18,700 ng of antibody protein per ml). These results suggest that there is not a direct correlation between anti-PRP antibody titer and functional capacity as measured by Hib opsonization.

Lack of correlation between protective level of Hib-specific antibodies and opsonic activity in children with recurrent Hib infection. Table 7 shows that 15 of 36 children vaccinated with Hib-PRP conjugate vaccine (42%) had protective levels of Hib-specific antibodies (i.e., ≥1,000 ng/ml) (8). Surprisingly, serum samples from 10 of the 15 children (67%) had poor or nonfunctional opsonic activities (subjects 4, 13, 16, 17, 20, 24, 26, 29, 31, and 34). Of the 10 children whose serum samples lacked opsonic activity, 5 (50%) presented with recurrent Hib infection.

Lack of correlation between titer of Hib-PRP antibody and opsonic activity in vaccinated children. Of the 21 children with postvaccination antibody concentrations below the protective level (i.e., <1,000 ng/ml), 11 (52%) showed opsonic activity above an OI value of 10 (Table 7) (subjects 1, 2, 5, 6, 8, 9, 10, 11, 19, 35, and 36).

TABLE 5. Establishment of interassay CVs for the anti-PRP assay with antibody concentrations[a]

Sample no.	Antibody concn (ng of protein/ml)		CV (%)
	Mean	SD	
1	757	99	13.0
2	404	34	8.0
3	210	20	10.0

[a] Interassay variability was represented by three samples tested in duplicate in 10 different runs.

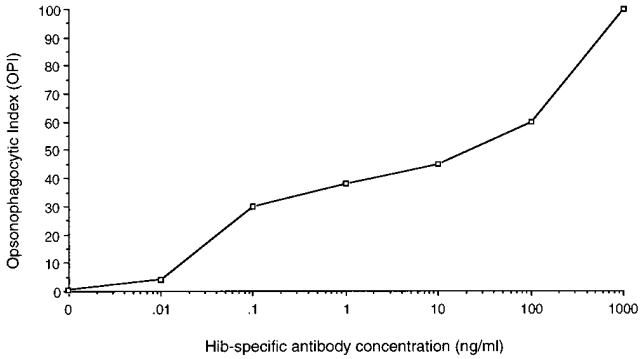

FIG. 1. Anti-Hib antibody dose dependence of the opsonophagocytosis reaction. Serum with a high anti-Hib titer (1:5,000) was used to opsonize Hib in a dose-dependent manner (0.01 to 1,000 ng/ml). The OI obtained for each of the six anti-Hib concentrations was related to the OI obtained for Hib incubated in HBSS only.

Lack of correlation between IgG subclasses and opsonic activity. Nineteen of the 36 serum samples from children were large enough (in volume) to allow measurement of IgG subclasses. The results of this group showed no association between deficiencies of one or more IgG subclasses and decreased opsonic activity (data not shown).

DISCUSSION

In this study, luminol-enhanced CL was used to develop a sensitive opsonization assay to study the interaction of heterologous neutrophils with Hib opsonized with sera from healthy adults and from children with recurrent Hib infection. The assay can be used for any other type of bacterial infection in which specific antibodies are present.

Our results indicate that high or low concentrations of anti-Hib antibody in circulation do not correlate with high, low, or no capacity for Hib opsonization. Furthermore, differences in serum IgG subclass concentrations did not explain the different

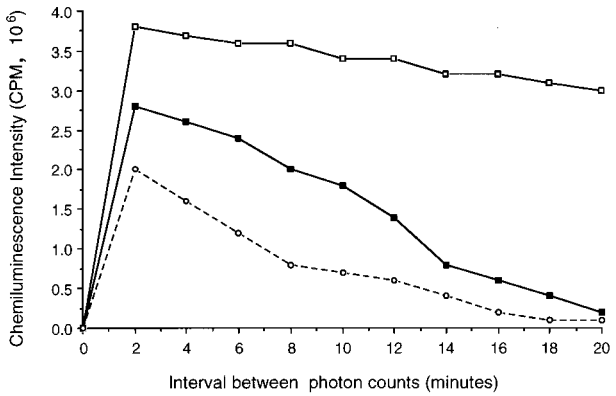

FIG. 2. Anti-Hib specific antibody-induced CL: differences in opsonic capacity of two serum samples. Two different serum samples with the same anti-Hib titer (1:900) were used to opsonize Hib to enhance phagocytosis by heterologous neutrophils. Symbols: □, highly functional serum; ■, moderately functional serum; ○, HBSS (blank = unopsonized Hib).

TABLE 6. Functional opsonic capacity of anti-Hib antibodies in healthy adults

Patient no.	Hib-PRP antibody level (ng of protein/ml)[a]	OI[b]
1	663	12
2	1,306	64
3	207	63
4	3,396	108
5	660	83
6	10,350	72
7	4,255	61
8	18,700	64
9	2,043	72
10	80,852	80
11	695	58
12	330	60
13	490	81
14	2,199	93
15	1,540	73
16	4,237	49
17	482	76
18	783	76
19	427	92
20	900	85

[a] The minimum protective level for unvaccinated individuals is 200 ng/ml.
[b] An OI below 10 represents nonfunctional opsonic activity.

opsonic capacities of the sera from the healthy adults or sick children.

It has been demonstrated previously that the avidities of antibodies from infants immunized with conjugate Hib vaccine strongly correlate with bactericidal activities of the anti-Hib sera (9). Although not tested by us, it is possible that the differences in functional capacities observed among the different serum samples (from 20 adults and 36 children) may be due to differences in antibody avidity. Indeed, low-affinity antibodies induce less-efficient phagocytosis, and poorly functioning IgG opsonins in the airway have been reported to contribute to chronic infections (7, 10). In our system, complement did not play a role because it was inactivated by treatment of the sera before analysis in the opsonization assays since the objective of the present study was to focus on antibodies alone. This assay will be used in future studies to evaluate the role of antibody and complement.

To assess the practical problem of defining an adequate response, both quantitative and functional measurements of humoral immune response to Hib may be necessary. Our data clearly show that some, but by no means all, individuals with a defective serum capacity for Hib opsonization as measured by neutrophil CL have recurrent infections. The evaluation of opsonic capacity of sera from patients with vaccine failure as reflected in Hib disease will be critical in the study of the clinical utility of opsonization by luminol-dependent CL. To this end, this rapid and sensitive method will be utilized to study a larger number of patients with Hib disease with defective serum opsonic capacity. The results of such studies may be essential for evaluating the decision-making process for treatment of Hib infections, including immunoglobulin replacement if functional impairment of the immune response is present.

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TABLE 7. Functional opsonic capacity of anti-Hib antibodies in children vaccinated with Hib-PRP conjugate vaccine

Patient no.	Hib-PRP antibody level (ng of protein/ml)[a]	OI[b]
1	472	13
2	389	89
3	876	3
4	4,725	14
5	535	28
6	531	16
7	509	5
8	439	65
9	584	65
10	266	64
11	523	100
12	6,558	43
13	4,599	4
14	730	2
15	197	0
16	1,156	5
17	2,517	2
18	495	1
19	590	36
20	1,904	17
21	3,521	47
22	1,019	37
23	1,212	47
24	2,326	34
25	2,052	0
26	1,008	1
27	323	2
28	122	0
29	1,444	11
30	407	3
31	1,132	3
32	182	2
33	877	0
34	1,393	0.9
35	125	44
36	34	48

[a] The minimum protective level for vaccinated individuals is 1,000 ng/ml.
[b] An OI below 10 represents nonfunctional opsonic activity.

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