Assessment of *Haemophilus influenzae* Type b Opsonins by Neutrophil Chemiluminescence

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A luminol-enhanced chemiluminescence assay and a radiolabeled uptake assay were developed to assess opsonins for *Haemophilus influenzae* type b. Opsonins in acute and convalescent sera from 17 children with *H. influenzae* type b meningitis, along with pooled normal human sera, were evaluated and compared with anti-polyribosephosphate antibody concentrations. Five children had a rise in the chemiluminescence-area under the curve for convalescent compared with acute sera. Patient chemiluminescence-area-under-the-curve values were significantly ($P < 0.05$) more likely to exceed 50% of normal human serum values if sera contained $\geq 0.1 \mu\text{g}$ of anti-polyribosephosphate antibody per ml. Magnesium ethylene glycol tetraacetic acid chelation and heat inactivation of patient and normal human sera significantly ($P < 0.05$) reduced chemiluminescence-area-under-the-curve activity. Thus, complement appears to contribute significantly to the opsonization of *H. influenzae* type b in sera of children. Two of nine children had increases in opsonins as assayed by ³H-labeled *H. influenzae* type b uptake. After natural systemic *H. influenzae* type b infection, young children are unable to respond acutely with an increase in anti-polyribosephosphate antibody or serum opsonic activity.

Haemophilus influenzae type b (HITB) is the most common bacterium responsible for bacterial meningitis in children less than 6 years of age. Antibody directed against the polyribosephosphate (PRP) capsule of HITB is thought to be protective against this organism (1, 7). Young children do not appear capable of producing an adequate or persistent antibody response to PRP after natural infection or immunization with purified PRP (3, 4, 13-15, 18).

In adults, an increase in serum anti-PRP antibody concentration generally results in increased serum opsonic activity for HITB. However, opsonic activity occurs in serum without detectable anti-PRP antibody, and thus antibodies directed against noncapsular antigens may be opsonic for HITB. Furthermore, both the classical and alternative complement pathways participate in the host defense against HITB in adult sera and in experimental models of HITB meningitis. Very little information is available about serum opsonins or the relative contribution of the complement pathways in the host defense system against HITB in children (3, 6, 7, 10).

Serum bactericidal activity interferes with the detection of serum opsonins for *H. influenzae*

type b when classic laboratory techniques that measure decreases in colony-forming units of preopsonized bacteria after the addition of leukocytes are used (12). The measurement of light emission or chemiluminescence (CL) by leukocytes which are phagocytizing opsonized bacteria has been shown to be an effective means for evaluation of serum opsonins for specific organisms such as *Escherichia coli* (1, 19, 20) and type III group B *Streptococcus* (2). We have designed a luminol-dependent CL assay for the assessment of opsonins to HITB in acute and convalescent sera of children with meningitis due to HITB.

MATERIALS AND METHODS

Study patients. Eighteen children hospitalized at Texas Children's Hospital with bacterial meningitis due to HITB comprised our study population. The ages of the children ranged from 5 months to 27 months. Informed parental consent was obtained before enrollment in the study. Paired sera were obtained within 24 to 36 h after admission and on the day of discharge. Blood samples were allowed to clot at room temperature and then placed in a refrigerated centrifuge at 4°C. Aliquots of sera were stored at -70°C until assayed. A normal human serum (NHS) pool was prepared from the sera of 11 healthy laboratory per-

sonnel.

***H. influenzae* isolates.** Clinical isolates of *H. influenzae* were identified by standard bacteriological methods and serotyped by countercurrent immunoelectrophoresis with HITB antisera (Hyland Laboratories, Deerfield, Ill.) (11, 23). The Z strain of HITB (isolated from a child with bacterial meningitis at Texas Children's Hospital) was grown in Todd-Hewitt broth supplemented with hemin and nicotinamide adenine dinucleotide (10 µg of each per ml; Sigma Chemical Co., St. Louis, Mo.) and added to 20% glycerol in tryptic soy broth (Difco Laboratories, Detroit, Mich.) before storage at -70°C. Other isolates that were used in this study were preserved in an identical manner. Before use in opsonic assays, two loops of thawed HITB suspension were inoculated into 10 ml of Todd-Hewitt broth plus hemin and nicotinamide adenine dinucleotide and incubated overnight at 37°C in a CO₂ incubator. Bacteria were washed three times in phosphate-buffered saline (PBS), pH 7.2, with 0.08% glucose and 0.25% bovine serum albumin and resuspended in 1.0 ml of PBS to achieve a final concentration of approximately 10¹⁰ colony-forming units per ml.

Serum treatment. (i) Inactivation of complement. Sera were decomplexed by heating at 56°C for 30 min. Sera were chelated with 10 mM/MgCl₂ and 10 mM ethylene glycol tetracetic acid (EGTA) (Sigma) to selectively inactivate the classical complement pathway (21).

(ii) Absorption experiments. Aliquots (1 ml) of pooled NHS were absorbed with the addition of 50 µg of PRP dissolved in 0.1 ml of normal saline and incubated for 30 min at room temperature and then overnight at 4°C (7). Unabsorbed sera were incubated in 0.1 ml of normal saline alone. Heat-inactivated pooled NHS also were absorbed with HITB from an overnight growth by incubating HITB in 1 ml of NHS for 45 min at room temperature and then overnight at 4°C. Bacteria were removed by high-speed centrifugation and membrane filtration. Unabsorbed control sera were treated in an identical manner without the addition of bacteria. Sera with nondetectable anti-PRP antibody were added to absorbed heat-inactivated sera to provide complement reconstitution.

Opsonization procedures. Untreated sera were diluted to 25% with normal saline since at that dilution the optimal CL response occurred with the least amount of sera. A 0.1-ml amount of washed bacteria in PBS was added to 1.0 ml of the previously prepared sera. The mixture was incubated for 30 min at 37°C in a shaking water bath, washed once in PBS, and resuspended to 1.0 ml.

Preparation of human leukocytes. Leukocytes were separated from whole blood by dextran sedimentation (9). Venous blood was obtained from the same healthy adult donor (S.L.K.) for each assay. Polymorphonuclear cells (PMN) were counted and resuspended in PBS to a final concentration of approximately 10⁶ PMN per ml with a Hycell Counter 300 (Hycel, Inc., Houston, Tex.).

Luminol-dependent CL assay. In preliminary experiments, we determined that a bacteria:PMN ratio of 1,000:1 and a luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) concentration of 10⁻⁷ M in

PBS were optimal conditions to allow evolution of CL activity during phagocytosis of HITB. Without luminol enhancement, CL responses could not be distinguished clearly from phagocytic mixtures containing no test organisms. A 1-ml amount of PMN suspension (10⁶ PMN) was added to a plastic scintillation vial containing 5 ml of 10⁻⁷ M luminol in PBS. After a 10-min base-line period, 0.1 ml (10⁹ colony-forming units) of opsonized HITB was added to the vials. The vials were placed immediately in a liquid scintillation counter (model C-2425; Tricarb, Packard Instruments Co., Downers Grove, Ill.) at ambient temperature with a coincidence circuit off in the L → U mode with 10% gain. Vials were counted sequentially for 1 min at 10-min intervals over 110 min. (Peak CL counts always were reached before this time period.)

When acute and convalescent patient sera were assayed, the protocol included PMN alone, HITB opsonized with pooled NHS as a control, and the patient samples. Untreated, heat-inactivated, and MgEGTA-chelated pooled NHS and acute and convalescent patient sera were run in the same assay. One pair of patient sera was evaluated each day along with the pooled NHS control. CL data is presented as (i) timed interval recordings of CL intensity in counts per minute versus time in minutes, and (ii) area under the curve (AUC) of the CL response between 20 and 110 min after the addition of bacteria calculated by the trapezoidal rule (1, 16, 20).

Radiolabeled uptake procedure. A modification of the radioactive-labeled phagocytosis assay of Mills et al. (9) was employed. The Z strain of HITB was prepared as in the CL experiments except 0.1 ml of ³H-amino acid mixture (1 µCi/ml; Amersham Corp., Arlington Heights, Ill.) was added to Todd-Hewitt broth. After overnight incubation of 37°C, labeled bacteria were washed three times in cold PBS and resuspended in 1.0 ml of Hanks balanced salt solution plus 0.1% gelatin at pH 7.2. Opsonization procedures were identical to the CL assay, except a 50% dilution of sera was used. Approximately 4 × 10⁶ PMN and 4 × 10⁹ colony-forming units of opsonized bacteria were placed in a plastic scintillation vial containing 4 ml of Hanks balanced salt solution-gel. The vials were placed in a water bath shaker at 37°C for 30 min. Aliquots (1 ml) were placed over 3-µm polycarbonate filters (25-mm diameter; Nucleopore Co., Pleasanton, Calif.) in a filter manifold (Millipore Corp., Bedford, Mass.). The filters were washed twice with 3 ml of PBS at 4°C and placed in 10 ml of Insta-Gel scintillation cocktail (Packard) and counted in a liquid scintillation counter.

For each test serum, opsonized bacteria without leukocytes also were incubated and filtered to determine nonspecific binding of the opsonized bacteria to the filter. Acute and convalescent sera from the same patient, in addition to pooled NHS, were run in each assay. Uptake of test sera was expressed as a percentage of uptake of pooled NHS. All determinations were performed in triplicate. The formula used for calculation of percent uptake was as follows: uptake, as percent of NHS = [(counts per minute for leukocytes + patient opsonized bacteria - counts per minute for patient opsonized bacteria alone)/(counts per minute

for leukocytes + NHS opsonized bacteria – counts per minute for NHS opsonized bacteria alone)] $\times 100$.

Anti-PRP antibodies were determined by radioimmunoassay as previously described (17). Significant increases in antibody concentration were defined as (i) a twofold or greater increase in convalescent versus acute sera, (ii) an absolute increase to greater than 0.1 $\mu\text{g/ml}$.

Statistical analysis. Statistical differences in CL data between patient acute and convalescent sera and pooled NHS were determined by the paired *t* test. Correlation coefficients were determined by linear regression analysis (5). In the CL-AUC results, a change was considered present if there was greater than a 10% difference between samples which exceeds the $t_{0.99}$ confidence interval determination for replicate samples. In the radiolabeled uptake procedure, a difference was considered to be present if there was a 50% or greater difference between two samples in an assay which approximates the $t_{0.95}$ confidence interval determination for replicate samples.

RESULTS

In this CL assay, PMN alone or PMN in the presence of unopsonized HITB resulted in no evolution of light emission compared with background counts.

To determine the intra-assay variation of the phagocytic CL response, nine replicate vials were run in a single experiment with PMNs and HITB Z strain. The mean $\pm$ standard deviation and coefficient of variation of the CL-AUC were $5.226 \times 10^6 \pm 0.154 \times 10^6$ counts and 3%, respectively. Inter-assay variation was determined in 18 separate CL assays with pooled NHS as opsonin and the same PMN donor with HITB Z strain. The mean AUC $\pm$ standard deviation and coefficient of variation were $3.96 \times 10^6 \pm 0.82 \times 10^6$ counts and 26%, respectively. The intra-assay and inter-assay coefficients of variation for the radiolabeled uptake procedure were 28 and 35%, respectively, based on nine separate assays with pooled NHS as the opsonin source included twice in triplicate for each assay.

Effect of different strains of HITB on CL evolution. To ensure that using a single strain of HITB in the phagocytic-CL assay would not affect the relative results of CL-AUC of acute or convalescent patient sera compared with pooled NHS, four different HITB strains were utilized as test organisms. As shown in Table 1, no differences were observed in the relative CL-AUC responses when different strains were used, although there were differences in absolute values. Thus, the same strain (Z strain) was used in all further opsonic CL assays to assess patient sera. The CL-AUC response with NHS containing ampicillin (Bristol Laboratories) or chloramphenicol (Warner Lambert/Parke Davis) at 20 $\mu\text{g/ml}$ was no different from NHS without

TABLE 1. Effect of different HITB strains on the opsonic activity assessed by CL

Serum	AUC (counts $\times 10^{-3}$) for strain			
	Z	35	74 ^a	55
Pooled NHS	4,056	5,405	3,141	3,063
Acute serum, patient 74	2,819 (69) ^b	3,559 (66)	2,572 (82)	2,376 (78)
Convalescent serum, patient 74	3,473 (86)	3,871 (72)	2,876 (92)	2,573 (84)

^a Strain 74 was isolated from patient 74.

^b In parenthesis is the percentage of pooled NHS.

added antibiotics (data not shown).

Dilution and absorption studies. To demonstrate the effect of anti-PRP antibody on opsonic CL activity, twofold dilutions of NHS (containing 3.3 μg of anti-PRP antibody per ml) in sera without detectable anti-PRP antibody were performed. A progressive decrease in CL-AUC activity was observed, indicating a positive correlation between serum opsonic activity for HITB and anti-PRP antibody concentrations (Fig. 1).

Absorption of NHS With PRP did not result in any significant change in the CL-AUC response. However, heat-inactivated NHS absorbed with PRP showed a significant decrease in opsonic CL activity compared with unabsorbed, heat-inactivated NHS (Fig. 2). Heat-inactivated NHS absorbed with HITB organisms and without added complement did not result in a decrease of AUC measurement as compared with heat-inactivated, unabsorbed NHS. When the same experiment was repeated after complement reconstitution with sera containing no detectable anti-PRP antibody, the CL-AUC of the unabsorbed NHS was much greater than that of the HITB organism-absorbed NHS (Fig. 3). In the absorption experiments, pooled NHS, PRP-absorbed pooled NHS, and whole-organism HITB-absorbed pooled NHS contained 2.9, <0.1, and <0.1 μg of anti-PRP antibody per ml, respectively.

Comparison of opsonic activity of pooled NHS and acute and convalescent patient sera. In Table 2, the means $\pm$ 1 standard deviation of the CL-AUC for pooled NHS and acute and convalescent patient sera are compared. The CL-AUC values of NHS were significantly ($P < 0.01$ to 0.001) greater than acute or convalescent sera in all categories by paired *t* test. Untreated acute patient sera had significantly greater CL-AUC activity than either MgEGTA-chelated ($P < 0.05$) or heat-inactivated ($P < 0.01$) acute patient sera. There was no significant difference between acute sera that was MgEGTA chelated or heat inactivated. Both untreated and MgEGTA-chelated convalescent pa-

FIG. 1. Interval recording of luminol-enhanced CL assay for HITB opsonins with twofold serial dilutions of pooled NHS. CL-AUC measurements are included with the temporal tracings.

FIG. 2. CL assay for HITB opsonins in NHS after absorption with 50 μ g of PRP or normal saline with and without heat inactivation. CL-AUC measurements are included with the temporal tracings.

tient sera had significantly greater ($P < 0.02$) CL-AUC values than heat-inactivated convalescent sera. There were no significant differences in CL-AUC responses between acute and convalescent sera which were untreated, MgEGTA chelated, or heat inactivated. When patient CL-AUC values were expressed as a percentage of NHS activity within the same assay, there was no correlation between the age of the patient and the percentage of NHS value in either acute or convalescent sera.

When individual pairs of acute and convales-

cent patient sera were compared, five patients showed a rise in CL-AUC values for convalescent compared with acute sera. In paired sera of 13 children, either no change occurred or the acute sera exceeded the convalescent sera in CL-AUC values (Table 3). Of 17 children, 5 had significant increases in anti-PRP antibody and 12 showed no changes when their convalescent sera were compared with acute sera. In three sera, anti-PRP concentrations increased significantly but CL-AUC measurements did not, whereas in two children CL-AUC measurements

FIG. 3. CL assay for HITB opsonins in heat-inactivated NHS after whole HITB absorption and with or without complement reconstitution. CL-AUC measurements are included with the temporal tracings.

TABLE 2. Comparison of CL-AUC response for NHS and patient acute and convalescent sera

Serum	Mean AUC $\pm$ SD (counts $\times 10^{-3}$) ^a		
	Untreated	MgEGTA chelated	Heat inactivated
Acute patient	2,175 $\pm$ 791	1,822 $\pm$ 903	1,812 $\pm$ 880
Convalescent patient	1,952 $\pm$ 748	1,837 $\pm$ 985	1,668 $\pm$ 794
Pooled NHS	3,196 $\pm$ 826 ^b	2,587 $\pm$ 1,059 ^c	2,552 $\pm$ 979 ^d

^a $n = 18$. SD, Standard deviation.

^b Pooled NHS > acute: $t = 5.338$, $P < 0.001$; NHS > convalescent: $t = 7.49$, $P < 0.0001$.

^c Pooled NHS > acute: $t = 3.52$, $P < 0.01$; NHS > convalescent: $t = 3.89$, $P < 0.001$.

^d Pooled NHS > acute: $t = 4.03$, $P < 0.001$; NHS > convalescent: $t = 5.70$, $P < 0.001$.

increased but anti-PRP antibody concentrations did not increase. In sera of two additional children, both CL-AUC and anti-PRP antibody concentrations increased. CL-AUC values were significantly ($\chi^2 = 3.8$, $P < 0.05$) more likely to exceed 50% of the NHS value if patient sera contained $\geq 0.1 \mu\text{g}$ of anti-PRP antibody per ml. However, 13 of 21 sera (62%) with nondetectable concentrations of anti-PRP antibody had greater than 50% of NHS CL-AUC values.

Paired sera from nine children were available for evaluation of opsonins by the radiolabeled uptake technique. Significant increases in ^3H -labeled HITB uptake occurred in sera from only two children, both of whom had significant in-

creases in anti-PRP antibody concentrations. Five children showed no increase in ^3H -labeled HITB uptake or anti-PRP antibody concentration, and in one additional pair of sera there was no rise in ^3H -labeled HITB uptake, but there was a significant increase in anti-PRP antibody concentration.

When expressed as a percent of NHS, the opsonic activity of heat-inactivated patient sera by CL-AUC correlated significantly ($r = 0.579$, $P < 0.05$) with opsonic activity as determined by ^3H -labeled HITB uptake (Fig. 4). In the sera of the two children (Table 3, patients 10 and 13) with an increase in ^3H -labeled HITB uptake, there also was an increase in CL-AUC activity.

DISCUSSION

Most investigators agree that antibodies directed against the capsular polysaccharide of HITB play an important role in host defense against this organism. Anderson et al. (3) have shown that anti-PRP antibody is related, in part, to the opsonic activity of serum for HITB. The opsonic activity of normal adult serum, as determined by a spectrophotometric Nitro Blue Tetrazolium dye technique, was related to the titer of HITB bactericidal and passive hemagglutination antibody. In serum absorbed with PRP, opsonic activity of post vaccination serum (with passive hemagglutination titers = 320) was decreased by approximately 50%. In sera from un-

TABLE 3. *HITB* opsonins and anti-PRP antibody

Patient no.	Age (mo)	CL-AUC (% pooled NHS)						Radiolabeled up-take (% NHS)		Anti-PRP antibody (μg/ml)	
		A ^a	C ^b	MgEGTA		Heat inactivated					
				A	C	A	C				
								A	C	A	C
1	5	72	76	66	80	65	74			<0.025	<0.025
2	5	68	46	93	75	116	72			0.16	0.066
3	8	88	29	89	64	63	55			<0.025	<0.025
4	10	61	77	98	90	77	81	72	54	<0.025	<0.025
5	11	114	85	59	102	90	87	99	97	<0.025	<0.025
6	11	56	71	44	44	71	69			0.025	0.06
7	11	67	44	103	90	80	74	103	81	<0.025	<0.025
8	12	45	47	37	45	56	58			0.033	<0.025
9	12	47	32	52	46	63	65	31	29	<0.025	0.04
10	13	85	79	103	87	71	80	37	59	<0.025	0.96
11	15	83	72	116	100	124	92	52	57	0.34	0.18
12	16	68	64	86	76	88	76	76	77		
13	16	66	83	93	96	54	64	0	67	<0.025	0.42
14	16	32	21	17	13	19	14			0.025	<0.025
15	17	36	48	35	48	31	33			<0.025	<0.025
16	17	109	67	77	60	85	60	106	95	<0.025	0.37
17	27	77	91	81	99	73	60			0.046	0.08
18	25	63	51	74	64	68	55			<0.025	0.84

^a A, Acute.^b C, Convalescent.

FIG. 4. Correlation of ³H-labeled *HITB* uptake with CL-AUC for assessment of *HITB* opsonins in children after *HITB* meningitis. Results of both assays are expressed as a percentage of the NHS evaluated concomitantly ($r = 0.579$, $P < 0.05$).

vaccinated adults, very little opsonic activity was absorbed with PRP. The authors concluded that opsonization of *HITB* can be effected

through antibodies directed against both PRP and non-PRP antigens (3). Granoff and Rockwell also demonstrated the importance of non-PRP-directed antibodies in infant rat sera with a radiolabeled uptake technique to measure opsonic activity (7).

The importance of complement in the host defense against *HITB* has been emphasized by several investigators. Heat-inactivated serum containing high-titer anti-PRP antibody demonstrated less opsonizing activity than did the same untreated serum specimen (8). In addition, C3-deficient sera were associated with decreased opsonic activity compared with normal adult sera (8). When infant rats were inoculated intranasally with *HITB*, the incidence and degree of bacteremia as well as the mortality rate were significantly greater ($P < 0.05$) in rats depleted of C3 and C5 (by cobra venom factor) than in control rats (6). In a modified phagocytic bactericidal assay, Newman et al. showed that in the presence of phagocytes, heated immune serum promoted much less killing than did fresh immune serum (12).

Opsonophagocytic CL assays have been developed previously for the detection of opsonins to various bacteria. Stevens and co-workers demonstrated deficiencies in the opsonic activity of serum from three of eight neutropenic leukemic patients with gram-negative septicemia (19). Opsonic deficiencies were noted for strains isolated from the patients or a laboratory strain

of *E. coli* or both. Anderson et al. compared a luminol-enhanced CL assay for the detection of opsonins for type III group B *Streptococcus* to both a radioactive antigen-binding assay for the measurement of type-specific antibody and a bactericidal assay (2). The CL activity of both untreated and heat-inactivated sera correlated significantly with antibody concentration. Anderson et al. stressed the importance of comparing the results of CL assays with other functional as well as nonfunctional assays when CL assays are used to assess serum opsonins (2).

In the present study, opsonins for HITB were assessed by a luminol-dependent CL assay and a radiolabeled uptake method and compared with anti-PRP antibody as measured by radioimmunoassay. Absorption of heat-inactivated pooled NHS with purified PRP resulted in a significant decrease in opsonic CL activity. Although absorption of fresh sera with PRP did not alter the CL measurements, absorption with HITB organisms resulted in a significant reduction of CL activity in complement-reconstituted sera. In addition, twofold sera dilutions of NHS with sera containing nondetectable concentrations of anti-PRP antibodies clearly decreased activity. Substantial CL activity remained in both the PRP or HITB-absorbed and diluted sera. Thus, with a CL assay, the opsonic activity from non-PRP-related antibodies can be detected in NHS. Heat-inactivation or MgEGTA chelation of NHS or patient sera resulted in significant decreases ($P < 0.01$) of CL activity compared with untreated sera. Thus, with the CL assay employed, complement appears to contribute significantly to the opsonization of HITB in sera of children as well as adults.

Children ≤ 2 years of age demonstrate a poor or absent immunological response to PRP after natural infection or immunization (13, 15, 18). In this study, 5 of 17 children had a significant increase in anti-PRP antibody concentrations for convalescent versus acute sera. Three of these children were ≥ 16 months of age. Five children had an increase in opsonic CL activity in convalescent sera when compared with acute sera. Three of these children also were ≥ 16 months of age. The remaining 13 children had no change or actually a decrease in opsonic CL activity in convalescent compared with acute sera. Only two of nine children assessed by the radiolabeled HITB uptake method had a rise in opsonic activity. Thus, in young children, the opsonic activity of sera obtained at the end of a systemic illness due to HITB usually is no different from that of sera obtained early during the course of the illness.

Since capsular PRP of HITB does not appear

to be an effective vaccine in young children for the prevention of disease due to HITB (15), other components of the HITB organism are being considered as alternative antigens for active immunization. Myerowitz and co-workers (10) noted that cell wall antigens of a strain of *Actinobacillus* cross-react with noncapsular antigens of HITB. Antisera against this strain of *Actinobacillus* increased opsonic activity for HITB and offered some protection against experimental HITB disease in infant rats. Granoff et al. (D. M. Granoff, R. Q. Warren, J. Lam, B. Burton, and J. W. Costerton, Abstr. Annu. Meet. South. Soc. Ped. Res., 1979, p. 808A) demonstrated increased opsonizing activity in rabbit antisera prepared to HITB ghosts (outer cell membrane preparations). These authors propose that noncapsular antigens of HITB ghosts potentially may be immunogenic and protective against HITB disease. However, our data suggest that young children after natural HITB systemic infection are unable to respond acutely with an increase in anti-PRP antibody concentrations or with increase in opsonic activity whether or not that activity was related to or independent of anti-PRP antibody.

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