INFECTION AND IMMUNITY, Dec. 1974, p. 1315-1320

Vol. 10, No. 6
Printed in U.S.A.

Opsonin System of the Group B Streptococcus

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Received for publication 15 July 1974

The opsonization by polymorphonuclear leukocytes of group B streptococcal serotypes associated with neonatal sepsis and delayed meningitis was studied. A specific BIa opsonizing antibody (not related to the antipolysaccharide typing antibody) was present in only 10% of the population tested. Serotype BIa was not opsonized in the absence of this specific antibody. BIa antibody specificity was demonstrated by macroagglutination and absorption with BIa streptococci and extracts, but not by gel diffusion. The binding of complement by the BIa opsonin increased the mean phagocytic activity by 60%; complement was manifested via the classic and/or alternate C3-related pathway, but seldom by both concurrently. Serotypes BIb, BIc, BII, and BIII were naturally and nonspecifically opsonized in 95% of the human and baboon sera or plasma tested. Although similar levels of opsonization were present in hyperimmune rabbit sera, heat inactivation and homologous bacterial absorption did not reduce the level of phagocytic activity in the rabbit, human, or primate groups studied. These immunological studies confirmed previous findings that serotype BIa presents a serious hazard for neonatal sepsis, with a nearly 100% mortality. Exclusive isolation of BIII in delayed meningitis suggests that ingestion and subsequent killing by polymorphonuclear leukocytes of type III may differ from the other serotypes.

The opsonization of bacteria is an important immunological defense mechanism (22, 28). The role of opsonins in neonatal bacterial infections has been studied primarily with respect to *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Serratia marcescens*, and *Pneumococcus* (*Diplococcus pneumoniae*) (5, 7, 21, 18, 28). The opsonins of the group B streptococci have received little attention, since most group B immunological studies have been for purposes of classification (5, 15, 16, 25, 26).

G. H. McCracken has encouraged detailed immunological investigations of the group B streptococcus because of their involvement in serious neonatal disease (19). There is evidence that two of the subtypes may be predominantly associated with two distinct disease syndromes (1, 2, 8). The association of BIa with acute sepsis and BIII with delayed meningitis suggested more than just a coincidental relationship. Klesius et al. showed that a BIa plasma factor, present in a small percentage of the population, was responsible for specific opsonization of the organism (13).

The opsonization mechanisms of the five group B serotypes in human and primate sera are described. Not only is the implication of opsonins and disease syndrome discussed, but special emphasis is placed on the role of complement in opsonization via the classic and alternate C3 pathways.

MATERIALS AND METHODS

Bacteriological methods. The group B serotypes (BIa, BIb, BIc, BII, and BIII) were from R. C. Lancefield's laboratory. Streptococci were preserved by desiccation in sand; later, they were placed in Todd-Hewitt broth (Difco) containing 5% defibrinated sheep blood and stored at −70 C. Bacteria streaked onto neopeptone infusion agar slants were used for repeated inoculations of 5 ml of Todd-Hewitt broth for phagocytosis assays. Larger preparations (2 to 5 liters) of group B cultures for extraction, absorption, or immunization purposes were inoculated from 30-ml broth cultures (5 ml of culture for each liter of broth). All cultures were incubated at 37 C for 18 to 24 h.

Source of sera and polymorphonuclear leukocytes (PMNs). The source of 55 human maternal, 24 human cord, and 13 baboon (*Papio cynocephlus*) bloods was previously described (13). In addition, 26 adolescent and adult male human sera were tested. Bloods were collected in heparinized tubes and assayed within 72 h. In the test procedure, PMN

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preparations from these specimens were added to the homologous sera. When serum specimens only were to be tested, fresh PMNs from either a single nonhuman primate or human source were feasible in most situations. Where blood group incompatibilities existed, agglutinins could be easily removed by heterologous absorption.

Antisera production and immunization methods. Specific hyperimmune rabbit sera to the group B serotypes were prepared as previously described (13). BIa vaccine, for the immunization of baboons, was prepared as follows. First, trypsin (0.1%) (Difco; 1:250) in Todd-Hewitt broth was added to an overnight BIa broth culture (vol/vol) and incubated at 37 C for an additional 2 h. The bacteria were killed by heating at 60 C for 45 min. A formalin vaccine was prepared by adding 0.5% formalin in phosphate-buffered saline (PBS) (pH 7.2) to an 18- to 24-h BIa culture (vol/vol) and incubated for an additional 72 h at 37 C. Vaccine preparations were washed three times in PBS, resuspended in sterile physiological saline, and adjusted to 0.5 optical density (OD) units at 540 nm. Each vaccine was inoculated into two adult baboons according to the following schedule: 0.5 ml intravenously on day 1, and 1.0 ml intravenously on days 3, 8, and 10. Blood specimens were obtained from the baboons on days 2, 7, 9, and 15.

Extraction methods. Before the BIa cells were extracted, they were washed three times in PBS and resuspended 1:4 in saline or distilled water. Lancefield's method was used to prepare acid extracts (14). A hot alkaline extract was prepared by adding 1 N NaOH to the cell suspension until a pH of 9.0 was obtained; the preparation was then boiled for 12 min (27). A hot water extract was prepared according to the procedure of Rantz and Randall by autoclaving a 25% suspension of cells in water (23). After all extracts were centrifuged at 10,000 $\times$ g for 20 min, the decanted supernatant material, containing the soluble antigens, was neutralized with either 1 N NaOH or 1 N HCl. NaCl was added to the hot water extract to create an isotonic medium.

Absorption procedures. Three milliliters of an 0.18-OD culture adjusted at 540 nm was centrifuged for 10 min at 2000 $\times$ g. Absorption was accomplished by adding 0.5 ml of serum to these cells and, after mixing and incubation at 37 C for 30 min, the mixture was centrifuged for 10 min at 2000 $\times$ g. The serum was decanted for later testing; the "sensitized" cells (those used in absorption) were either discarded or washed three times in saline and tested for increased or decreased susceptibility to phagocytosis.

The absorption with soluble antigen was accomplished by adding 0.2 ml of undiluted extract or culture supernatant to an equal volume of test serum and incubating the mixture at 37 C for 30 min.

Agglutination and immunodiffusion methods. The slide agglutination procedure used in this study was previously described (13). The macroagglutination procedure was done by using plastic, disposable tubes (13 by 100 mm) essentially as described by Kabat and Mayer (12). One milliliter of a group B bacterial suspension, adjusted to an OD of 0.32 at 540 nm, was added to 1 ml of serum diluted 1:5 in PBS. After the contents of the tubes were mixed, the tubes were placed in a 37 C water bath for 2 h and then refrigerated at 4 C overnight. The tubes were centrifuged at 2,000 $\times$ g and read for agglutination. The microtemplate immunodiffusion procedure of Zimmerman et al. was used for the gel studies (30).

Phagocytosis methods. The procedure of Klesius et al., with 3% gelatin separating the leukocytes from the erythrocytes, was used (13). The leukocytes were washed twice with 5% bovine serum albumin in PBS and adjusted to approximately 12,000 cells per ml. The test mixture was 0.5 ml of leukocytes plus 0.2 ml of test serum plus 0.1 ml of the bacterial suspension (0.18 OD at 540 nm), and the ratio of bacteria to PMNs was about 300:1. The mixture was incubated at 37 C in a shaking water bath for 30 min, and duplicate slip smears were made. The slides were stained with Wright stain, and 50 mature (segmented) PMNs were randomly observed; the number of PMNs containing streptococci was recorded, and the percentage of phagocytic activity (PPA) was calculated (13).

Preparation of complement "R" reagents: R1 and R2. Sera deficient in C1 (R1) and C2 (R2) activity were prepared by inactivation at 56 C for 15 to 30 min (3, 12). Since there is recent evidence for inactivation of critical C3 factors by heating, the inactivation time at 56 C was 15 rather than 30 min (29).

R4. C4 was inactivated by NH_3 and KSCN treatment (4, 12). For ammonia treatment, 0.25 ml of 0.15 N NH_4OH was added to 1 ml of serum; this was incubated at room temperature for 1.5 h and neutralized with 0.25 ml of 0.15 N HCl. KSCN treatment consisted of slowly adding cold (4 C) KSCN (1 M) in water (vol/vol) to a manually agitated serum. This was kept at 4 C for 18 h and then dialyzed first against PBS and then Hanks balanced salt solution to restore Ca^{2+} and Mg^{2+} ions.

R3. C3 was inactivated by the above KSCN treatment. C3 was also inactivated by using cobra venom (*Naja naja*) (20). One volume of 0.5% cobra venom in PBS was added (vol/vol) to all BIa-positive sera and incubated at 37 C for 30 min.

All reagents were either used immediately after preparation or stored at −20 C.

RESULTS

Differences in opsonization levels between the five group B serotypes. The capacity for PMNs to phagocytize the five group B streptococcal serotypes in the presence of sera or plasma from normal human maternal, cord, and nonhuman primates was previously described (13). Twenty-six adolescent and adult male human sera were included in these data. When these sera (N = 118) were tested against serotypes BIb, BIc, BII, and BIII, their mean PPAs were similar ranging from 47.9 to 61.5. In contrast, the same sera had a considerably lower mean PPA (11.5) with serotype BIa. This

difference in PPA against BIa and the other serotypes is further represented in Table 1. Of the 101 human sera tested, 90 had PPA against BIa at a level lower than 25, whereas ≥94 of the same sera tested against BIb, BIc, BII, and BIII had PPA values ≥25.

Evidence for a specific BIa opsonizing antibody. We had previously shown that BIa opsonic activity could be removed by homologous but not by heterologous absorption of primate sera with whole cells (13). Further absorptions of additional BIa-positive human, baboon, and hyperimmune rabbit sera also removed the BIa opsonin. The "sensitized" BIa cells, even after several washings, would be readily phagocytized in the presence of diluent and PMNs.

Additional evidence for the antibody-like nature of the Ia opsonin was the solubilization of the corresponding antigen by various extraction methods (Table 2). Hot water and hot alkaline extracts along with the BIa culture supernatant removed the opsonin as well as or better than the whole BIa cells or hot acid treatment. We have previously reported that BIa cells would not agglutinate in the presence of BIa opsonin-positive sera when a slide agglutination technique was used (13). This was confirmed with the additional BIa sera used in this study; however, when a classic macroagglutination procedure was used, all of the BIa-positive human sera were positive for agglutination with BIa cells, but not with the other four serotypes. Gel diffusion showed the absence of grouping and typing carbohydrate antibodies in these sera. Immunodiffusion of Ia-soluble antigen preparations, containing the BIa opsonic antigen, against strongly positive BIa opsonin sera did not develop any precipitin bands.

Immunization of baboons with serotype BIa. Most of the BIa-positive sera in this study were from sources with naturally occurring BIa opsonic ability. Therefore, four baboons, negative for BIa opsonic activity, were inoculated with BIa streptococci to (i) determine the time Ia opsonin appeared after infection; (ii) determine whether BIa vaccines treated with trypsin or formalin would elicit different quantitative BIa responses; and (iii) confirm the antibody-like nature of the BIa opsonin (Table 3). Baboons no. 377 and 402 received trypsin-treated cells and baboons no. 363 and 01 were injected with formalin cells. All four baboons had minimal BIa activity on day 0 and considerable PPA with the heterologous BIII bacteria. The baboons inoculated with the trypsin-treated BIa cells developed PPA much earlier than the formalin-treated baboons. No. 377 showed significant activity on day 2 and no. 402 on day 7. Both no. 363 and 01 did not develop BIa PPA until day 15, and the BIII heterologous response did not change significantly during the experiment.

TABLE 1. *Individual differences in opsonization of the five group B serotypes*[a]

PPA value	Group B serotype									
	Ia		Ib		Ic		II		III	
	No. of sera	%	No. of sera	%	No. of sera	%	No. of sera	%	No. of sera	%
≥25	11	11	98	97	96	95	94	93	96	95
<25	90	89	3	3	5	5	7	7	5	5

[a] $N = 101$.

TABLE 2. *Effectiveness of various extraction methods to solubilize the Ia opsonin inhibitor*

Positive Ia human serum	Extract or other prepns used for absorption	Phagocytic system	Ia phagocytosis (%)
J.M.	None	Ia	84
J.M.	100 C acid	Ia	42
J.M.	50 C acid	Ia	64
J.M.	Hot water	Ia	12
J.M.	Hot alkaline	Ia	4
J.M.	Culture supernatant	Ia	0
J.M.	Whole cells	Ia	12

Role of complement in the opsonization process. Results of our previous study showed that inactivation at 56 C destroyed the PPA of the BIa serum factor (13). Later studies on positive BIa sera have shown an intricate relationship between the BIa opsonic antibody and complement via the classic and alternate C3 pathways.

The role of complement in the phagocytic response was evaluated by the inactivation of selected components through preparation of R reagents from every serum or plasma tested. The experimental rationale is shown in Fig. 1. "E" (BIa bacteria) added to "A" (BIa opsonizing antibody) and complement would give the maximal amount of opsonization in the uninterrupted condition. R1-2- and R4-treated sera would have no complement via the classic pathway, since 1-4-2 had been inactivated. Therefore, the alternate C3 pathway, first described by Gotze and Muller-Eberhard, and antibody would account for the remaining PPA (9). The preparation of R3 would interrupt both

TABLE 3. *Phagocytic response of baboons immunized with group B Ia streptococci*

Baboon	System	PPA					
		Pre-immune PPA	Days after immunization				
			0	2	7	9	15
377[a]	Ia	6	0	76	66	88	92
	III	48	36	68	62	74	86
402[a]	Ia	4	0	0	68	82	76
	III	48	54	48	76	54	70
363[b]	Ia	6	0	4	6	0	40
	III	42	30	52	62	50	56
01[b]	Ia	0	0	2	6	4	52
	III	60	52	48	62	74	76

[a] Immunizing agent: heat-killed trypsinized cells.
[b] Immunizing agent: formalin-treated cells.

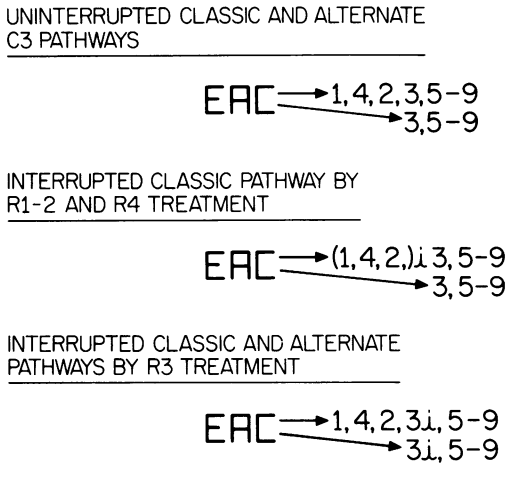

FIG. 1. *Inactivation of the classic and alternate C3 pathways with R reagents. Abbreviations: E, BIa bacteria; A, BIa opsonizing antibody; i, inactivated complement component(s).*

pathways, thereby eliminating the contribution of complement but not of antibody alone. Once the C3 pathway is interrupted, then so is the classic pathway, but not vice versa.

Figure 2 shows the mean combined PPA of the 18 positive human, baboon, and rabbit BIa sera used in this study. Untreated sera had a PPA value of 78.8. Inactivation with the appropriate R reagents showed that the classic pathway was responsible for 30.6 of the PPA, the alternate C3 was responsible for 18.8, and antibody contributed only 30.6 to the total PPA picture. These results show the importance of complement as an important adjunct to the total BIa opsonization reaction.

The role of these complement factors is even more striking when 10 of the 18 positive BIa sera were evaluated for PPA (Table 4). All untreated sera selected had PPA values ranging from 40 to 100. Antibody alone contributed more than half of the PPA in only 3 of the 10 samples and had no effect in two sera. The classic pathway was responsible for more than half of the PPA in 4 out of the 10 samples, and the C3 pathway, in 2 of 10: the high levels of PPA were primarily due to the added effect of complement.

When R reagents R1-2 and R4 were mixed, or added to R3, nearly complete PPA was restored. Adding fresh serum, negative for BIa opsonic antibody, also restored activity to the R reagents. This provided evidence for complement-dependent opsonization and satisfactory "R" reagents.

Opsonization of group B serotypes BIb, BIc, BII, and BIII. The opsonic nature of positive human and primate sera with respect to types BIb, BIc, BII, and BIII was determined. First, inactivation at 56 C for 20 min did not decrease the PPA of these sera; second, absorp-

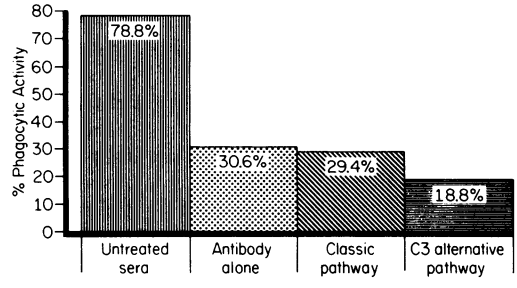

FIG. 2. *Cumulative effect of antibody and complement via the classic and alternate pathways on BIa PA (N = 18).*

TABLE 4. *Contribution of antibody and complement to the Ia opsonic activity in 10 sera*

Sera or plasma	Untreated	Classic pathway	Alternate C3 pathway	Antibody
J.M.[a]	94[b]	50[b]	18[b]	26[b]
J.W.[a]	82	30	36	16
R.T.[a]	100	100	0	0
B3[c]	68	8	0	60
367[d]	92	0	56	36
387[d]	88	0	36	52
01[d, e]	76	49	4	24
363[d, e]	40	2	38	0
377[d, e]	92	8	36	48
402[d, e]	76	60	0	16

[a] Human specimen.
[b] Percentage of phagocytic activity.
[c] Rabbit specimen.
[d] Baboon specimen.
[e] Vaccinated with BIa cells.

tion with homologous bacteria did not affect the PPA nor were the sensitized bacteria phagocytized at a greater rate than untreated bacteria when tested in 5% bovine serum albumin; third, hyperimmune rabbit sera to these four types were checked for PPA as shown in Table 5. Two of the four sera, BIb and BII, had low levels of homologous PPA before absorption; however, none of the four antisera showed any significant decrease after homologous absorption. Opsonization appeared to be nonspecific.

TABLE 5. *Effect of absorption of group B rabbit antisera with homologous bacteria before testing in phagocytosis*

Rabbit no.	Immune sera type	Absorption	Phagocytosis system	Phagocytic activity (%)
B5	Ib	None	Ib	12
B5	Ib	Ib	Ib	8
B14	Ic	None	Ic	68
B14	Ic	Ic	Ic	86
B10	III	None	III	50
B10	III	III	II	40
R117	II	None	III	14
R117	II	II	II	8

DISCUSSION

In a recent report, Winkelstein discussed the complexity of the opsonization process, due primarily to the interaction of specific antibody and complement to varying degrees (28). Opsonization can occur with antibody alone; it can occur with antibody and complement via the classic 1-4-2 pathway or via the alternate C3 pathway. Natural immunity may be another factor along with specific receptor sites on leukocytes as mediators of the opsonization process (22, 28). Because of this plethora of possibilities, phagocytic results, with respect to the mechanism involved, were indeed difficult to interpret (28).

The opsonization processes involved in the group B streptococci are also complex. Most bacterial opsonizing antibody is directed to polysaccharides located on the cell wall (22, 28). This seems to be the case with the BIa-specific opsonizing antibody, since trypsin and pepsin treatment did not affect the phagocytic resistance of the BIa organism (unpublished data). Therefore, the BIa opsonic antibody may be antipolysaccharide in nature. A low concentration of this antibody in positive sera may account for its absence in gel diffusion, although it was detected in the more sensitive macroagglutination procedure. Electron microscopy studies in our laboratory have shown the presence of long pili or fimbriae on the BIa subtype; these are either absent or in a diminished form on the other subtypes (C. B. Cropp, MSM thesis, Colorado State University, Fort Collins, 1973). Although the pili on the group A streptococci can be removed by trypsin, the same treatment does not affect the BIa pili, suggesting a nonprotein structure (24).

Group BIa opsonic antibody is a more efficient opsonin if it binds complement. Since Johnston showed that the action of complement via the classic pathway in opsonization is similar to the hemolytic action sequence of complement, we used R1-2 procedures to interrupt this pathway (11).

The alternate pathway was interrupted by using cobra venom. Briefly, a factor in cobra venom (CoF) combines with a serum protein called C3 proactivator (C3PA). This bimolecular complex becomes an active enzyme similar to C3 convertase. The CoF-C3PA complex cleaves C3 into C3a and C3b, mimicking the yet undefined C3PA convertase by activating the alternate C3 pathway. Recently, several investigators (6, 10, 29) have studied the participation of the alternate pathway in opsonization. This pathway has also been considered analogous to properdin (17). Jasin showed that adding purified C3PA to sera increased the phagocytic response (10). These results are not inconsistent if one considers that C3PA may lose opsonic prowess after complexing with cobra venom.

The immunological response of the human or other primate host with respect to the development of the BIa-specific opsonin and complement factors was vague, since most sera were selected by chance and without a known history of streptococcal experience. We do know that the BIa opsonin passes the placental barrier, since the cord sera of all BIa-positive maternal sera were positive. This is consistent with results on similar opsonin levels in maternal and cord sera (5, 18). The immunization of baboons with formalin and trypsinized BIa vaccines showed a later development of the BIa-opsonizing antibody in the group receiving the formalin-treated vaccine. Although trypsinization did not affect the pili as shown by electron microscopy, perhaps BIa antigenic sites on the pili were exposed that were otherwise masked. The complement pathway used could not be associated with the inoculation regimen. In one baboon from each group, the classic pathway predominated; in the other baboons alternate pathways predominated. However, in any single baboon only one or the other of the complement pathways developed.

The opsonization mechanism(s) involved

with BIb, BIc, BII, and BIII were different from that with BIa. These (BIb, BIc, BII, and BIII) serotypes were opsonized nonspecifically by 95% of the normal sera tested (N = 101) at high PPA levels without the aid of complement. Infant mortality rates associated with infections by these serotypes are also much lower than those caused by BIa (8). The exclusive implication of BIII in the meningitis syndrome may not be related to the opsonization process, but to the killing of the organism after ingestion by PMNs. Quie has shown that the killing by leukocytes of gram-positive organisms depends upon the production of hydrogen peroxide by that bacterium as a cofactor in, first, halogenation and, then, in the killing event (22). Therefore, BIII may be a poor hydrogen peroxide producer and thus may resist killing. This would fit the epidemiology of the delayed meningitis syndrome.

The data presented show the potential hazard for acute sepsis in the early neonate with serotype BIa, since opsonization will occur only in the presence of a specific antibody. This antibody was present in a small percentage of the population (10%), and the binding of complement by this antibody greatly enhanced the degree of opsonization.

ACKNOWLEDGMENTS

We would like to thank George H. McCracken, Jr., for reviewing and making helpful suggestions during the preparation of this manuscript. Thanks also go to Donald H. Krushak and associates for conducting the baboon experiments.

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