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Strain Specificity of Opsonins for Group B Streptococci Types II and III

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Strains of types II and III group B streptococci do not appear to be uniformly susceptible to opsonization by antibody-containing human sera, as studied using both a chemiluminescence and a radiolabeled bacterial uptake technique. We could not demonstrate a correlation of serum-sensitive or resistant strains with capsular antigen quantities, although serum absorption studies with whole organisms and HCl, trichloroacetic acid, and saline extracts indicated that the antibody to type-specific capsular polysaccharide is important in opsonizing both serum-resistant and serum-sensitive strains. Since trypsin treatment produced significantly enhanced opsonization of serum-resistant and serum-sensitive strains, proteins present on some group B streptococci may be important antiphagocytic factors.

Group B streptococci are a major cause of morbidity and mortality in neonates and in other patients with compromised host defense mechanisms (2, 5, 6, 8, 10, 12, 25). In previous studies, we have shown that patients who develop group B streptococcal sepsis usually lack serum opsonic activity for their infecting strain or for homologous type reference strains (9). It became apparent early in these studies, however, that strain-specific differences in opsonization occur. Not all strains of types II and III group B streptococci appear to be uniformly susceptible to opsonization by antibody-containing human sera. In this study we have investigated the prevalence of opsonins in normal adult human sera to different strains and have attempted to further characterize strain-specific antigens utilizing studies of serum absorbed with whole bacteria or HCl, trichloroacetic acid, and saline extracts. In addition, treatment of bacteria with trypsin and neuraminidase was used in attempts to modify possible bacterial surface antigens. Strain-specific differences in opsonization requirements for group B streptococci of types II and III may be of utmost importance in studies designed to test the protective efficacy of type-specific polysaccharide vaccines.

MATERIALS AND METHODS

Preparation and opsonization of organisms. Reference strains of group B streptococci type II (18RS21) and III (D136C) or group B streptococcal strains isolated from infected or colonized patients (wild strains) were cultured at 37°C in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) for 10 to 12 h. Of the 13 wild strains for which data are presented in Tables 1 and 2, 11 were isolated from the blood of infected infants (cerebrospinal fluid isolates of the same group B streptococcal type were available but not examined in three cases); one resistant and one sensitive strain (both type II) were skin isolates. Susceptibility to serum opsonic activity could not be correlated with site of isolate, whether the patient was infected, or patient mortality utilizing these and other strains examined in our laboratory. Relatively few strains from colonized patients have been obtained, however. In selected experiments, the strains were grown in the buffered, glucose-enriched broth described by Baker and Kasper (3). The bacteria were washed and concentrated in phosphate-buffered saline (PBS) by centrifugation to contain 5.0×10^8 to 1.0×10^9 colony-forming units per ml, as previously described (9, 23). These suspensions were opsonized with test serum in a ratio of 0.5 ml of bacteria to 0.1 ml of serum at 37°C for 30 min (9). This ratio was used because chemiluminescence (CL) results showed no significant change with the higher serum concentrations of 50 and 80% tested. The bacteria were then washed twice in PBS and readjusted to the original concentration, and used immediately in the CL procedure.

Serum treatment. Fresh serum or serum frozen at −70°C (thawed only on use) was used. To standardize the contributions of both complement pathways, all sera were heat inactivated at 56°C for 30 min. A volume of whole blood complement (0.025 ml, 50% hemolytic complement of 40, C3 of 71, C4 of 20, C5 of 9, Factor B of 15; Cordis Laboratories, Miami, Fla.) previously determined to maximally enhance the opsonic activity of heated human sera was then added (9).

Preparation of PMNs. Heparinized blood (10

U/ml) was collected from healthy adult volunteers, gravity sedimented to remove erythrocytes, and washed twice with PBS. Polymorphonuclear leukocytes (PMN) were then counted and adjusted to a concentration of 10^7/ml, representing 50 to 70% of the cells in the suspension. No PMN donor was used with autologous serum. Ten different PMN donors were utilized, and comparable CL levels were obtained. The same opsonizing serum tested on three different occasions had mean peak CL with a standard deviation that averaged 7 ± 6% of the mean (mean variance 6 ± 6% of the mean) (9).

CL procedure. Scintillation counting was performed as previously published (9, 23) in a Beckman LS100c liquid scintillation system (Beckman Instruments, Inc., Fullerton, Calif.) out of phase, with one photomultiplier tube disconnected. PMN (0.5 ml) and 0.5 ml of the bacterial suspension were mixed, and the volume was adjusted to 3.5 ml with sterile PBS. The vial was capped, immediately placed in the scintillation counter, and counted at approximately 10-min intervals for a total of 100 min. The CL detected by the counter is expressed in counts per minute. CL levels of ≥6,500 cpm correlated with deposition of immunoglobulin G on the bacterial surface by indirect immunofluorescence (23) and with phagocytic uptake by a radiolabeled bacterial uptake technique (R value of 0.79) (C. D. Allred, A. O. Shigeoka, and H. R. Hill, J. Immunol. Methods, in press) and visual examination of stained smears (23).

Preparation of group B streptococcal antigen. Hot HCl and cold trichloroacetic acid extracts of the reference strains of group B streptococci were prepared by slight modifications of the procedures of Lancefield and Freimer (15, 16, 18). PBS extracts were prepared as described by Kane and Karakawa (13). All extracts were examined for estimation of quantities of capsular polysaccharide antigen extracted by capillary precipitin technique and by counterimmunoelectrophoresis.

Capillary precipitin procedure. The capillary precipitin tests were performed by mixing equal volumes of rabbit hyperimmune antiserum and the antigen to be tested in sterile capillary tubes (16). Typing reactions were read in 10 min; formation of a precipitate was interpreted as a positive test. Varying dilutions of the antigen mixtures were used in an effort to roughly quantitate the type-specific antigen present. The final dilution of an extract yielding a precipitin band with a standard serum was used as an estimate of the relative quantity of the antigen present in the undiluted extract.

Counterimmunoelectrophoresis of group B streptococci. Counterimmunoelectrophoresis was performed by a procedure previously described (11). Briefly, glass microscope slides were covered with 1% agarose in barbital buffer, and parallel rows of wells were cut so that 2 mm separated antigen and antiserum. A standard type-specific rabbit antiserum in 10-μl portions was added to the well nearest the anode, and the antigen (from HCl, trichloroacetic acid, or saline extracts) was added to the well nearest the cathode. Electrophoresis was carried out at room temperature for 30 min using 5 to 7 mA per slide in a Gelman electrophoresis apparatus. The final dilution of an extract yielding a precipitin arc with a standard serum was used as an estimate of the relative quantity of the antigen present in the undiluted extract.

Treatment of bacteria with trypsin and neuraminadase. Bacteria were grown as described above with the addition of trypsin (1 mg/ml; Worthington Biochemicals Corporation, Freehold, N.J.) or neuraminidase (0.8 U/ml; Behring Diagnostics, American Hoechst Corp., Somerville, N.J.) over a 10- to 12-h period. In addition, washed bacteria were treated with trypsin or neuraminidase at the same concentrations for 0.5 and 3 h at 37°C, respectively. Bacteria were then centrifuged and washed three times before opsonization.

Absorption studies. Serum absorption studies were performed with preparations of washed, live whole organisms of several strains of types II and III group B streptococci as well as with saline, HCl, and trichloroacetic acid extracts of the same strains (13, 15, 16, 18). Absorption studies were carried out in a ratio of 1 volume of bacteria to 1 volume of serum at 37°C for 1 h with heated serum. This ratio was chosen arbitrarily, although other investigators (19) have used a 1:3 ratio for absorption studies with group B streptococci. Four studies performed with serum absorption repeated three times showed no further changes in CL levels or by radiolabeled bacterial uptake. In addition, studies were also performed at 4°C for 1 h; no difference in absorption at these temperatures was detected. Absorbed serum was negative for antibody by capillary precipitin and counterimmunoelectrophoresis in all instances.

Radiolabeled bacterial uptake technique. Bacteria were prepared by incubation in Todd-Hewitt medium containing [^{3}H]leucine (5 μCi/ml) at 37°C for 24 h, heat killed, washed three times in PBS, and adjusted to 3.3×10^8/ml (23; Allred et al., in press). Samples of bacteria were frozen at −20°C before use and at that time opsonized at 37°C for 30 min as described above. Opsonized bacteria were washed once and resuspended to the original volume in medium 199 (Microbiological Associates, Walkersville, Md.). Phagocytic uptake by human PMN was determined by a modification of the method previously described by Mandel (21). Monolayers of PMN were prepared by incubating 0.3 ml of leukocyte suspension on glass cover slips (18 by 18 mm) at room temperature for 30 min. Nonadhering cells were washed off with PBS, and 0.3 ml (10^8 organisms) of the opsonized, labeled bacterial suspensions was added to each cover slip. Control suspensions contained bacteria alone and unopsonized bacteria plus PMN. At intervals of 1, 10, 20, 30, 40, and 50 min cover slips were washed with PBS, placed in scintillation vials with 5.0 ml of Aquasol, and counted for 5 min in a Beckman LS-100c scintillation counter. The monolayers appeared evenly distributed by microscopic examination. The number of adherent cells (5×10^5 to 1.5×10^6) was estimated by counting cells in five random grids at 400 power and multiplying the average by the number of grids (20,227) per cover slip. The accumulated counts per minute per 10^6 adherent PMN was calculated at each interval and plotted against time. Controls with bacterial suspensions added to cover slips without cell monolayers or with cells but unopsonized bacteria showed no accumulation of radioactivity.

Gel diffusion methods. Group B streptococci

grown as described above were washed twice and resuspended in PBS (pH 7.4) to a turbidity of 350 Klett units (blue filter, Klett-Summerson Colorimeter). A 6-ml sample of this suspension was centrifuged, and the pellet was resuspended in 25 μl of sodium dodecyl sulfate solubilizing solution (14). After the sodium dodecyl sulfate-bacterial mixture was boiled for 10 min, the lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (14). A 10% polyacrylamide (polyacrylamide-BIS = 30:0.8) separating slab gel was used, and no sodium dodecyl sulfate was incorporated into either this or the 4% stacking gel (M. Wycoff, R. Rodbard, and A. Chrambach, Fed. Proc. 35:1383, 1976). Electrophoresis in tris(hydroxymethyl)-aminomethane-glycine (pH 8.3) buffer was carried out at 40-mA constant current. On completion of electrophoresis, the gel was removed, fixed overnight in 7% acetic acid-25% isopropanol, stained with 0.2% Coomassie blue R in acetic acid-isopropanol for 1 h, and destained by diffusion in acetic acid-isopropanol.

TABLE 1. *Prevalence of serum opsonic activity for clinical isolates of type II and III group B streptococci*

Type	Strain isolate	No. of sera tested	Sera with opsonic activity	
			No.	%
II	G	30	2	7
	VH	15	2	13
	MY	15	1	7
	HO	30	3	10
	WA	20	18	90
	MZ	15	12	80
III	H	45	2	4
	BE	35	1	3
	MA	15	1	7
	WI	18	6	33
	SE	35	35	100
	LA	16	13	81
	T	16	16	100

RESULTS

Strain-specific differences in opsonic activity. The CL and radiolabeled bacterial uptake procedures were used to systematically examine a number of adult sera for opsonins to multiple strains of type II and III group B streptococci isolated from infected infants. Some strains of each type were rarely opsonized by any of the sera examined (Table 1). For instance, only 2 of 30 sera examined opsonized isolate G. Other type II and III strains had a similar pattern of resistance to opsonization. Such strains were considered resistant or R strains. In contrast to these isolates, other strains were opsonized by the majority of sera examined; for instance, isolate WA was opsonized by 18 of 20 sera examined. These strains were considered sensitive or S strains. "Sensitive" and "resistant" are used in this paper to describe bacterial susceptibility to serum opsonic activity, not in the classical usage in reference to serum bactericidal or antibiotic sensitivity.

Table 2 demonstrates the strain-specific differences in opsonic activity in adult human serum. Some sera possessed excellent activity against one strain of a given type but completely lacked opsonins to another isolate of the same type. For example, serum sample 1 contained opsonins for isolates VH, WA, and MZ, but not for three other isolates of type II group B streptococci.

Utilization of a radiolabeled bacterial uptake technique confirmed the differences in opsonization of sensitive and resistant strains of these group B streptococci. Both the CL and radiolabeled bacterial uptake techniques were used to examine opsonins to sensitive and resistant strains of type II and III (i.e., four separate strains) for each serum sample. The radiolabeled uptake technique was not used to confirm all CL results, since the two assays correlated well (R value 0.79) (Allred et al., in press). Figure 1 shows the difference in uptake when a serum containing heat-stable opsonins to only a type III-sensitive strain was used to opsonize a type III-resistant strain. This serum possessed immunoglobulin G antibody to type III group B streptococci as determined by indirect immunofluorescence.

Enhanced capsular polysaccharide antigen production. To determine whether a difference in quantity of capsular polysaccharide antigen could account for the observed strain variation in opsonization, several strains of resistant and sensitive group B streptococci were grown in buffered glucose-enriched Todd-Hewitt medium described by Baker and Kasper (3). There was little difference in opsonization after this manipulation; increased capsular polysaccharide did not prevent or decrease serum opsonization of any strain by the CL procedure or radiolabeled bacterial uptake technique. In fact, some group B streptococci grown in this medium showed slightly higher CL values compared to the same strain grown in regular Todd-Hewitt medium and opsonized with the same serum. Bacteria grown in this medium did appear to produce somewhat larger amounts of type-specific polysaccharide antigen as indicated by the final dilution of extracts reacting in capillary precipitin and counterimmunoelectrophoresis studies. Similar experiments failed to show any correlation between type-specific capsular antigen production and whether a strain was sensitive or resistant to opsonization by serum (Table 3).

Absorption studies with whole orga-

TABLE 2. *Strain variation in serum opsonic activity for clinical isolates of type II and III group B streptococci*

Type	Strain isolate	Serum sample[a]											
		1	2	3	4	5	6	7	8	9	10	11	12
II	G	−	−	−	−	+	−	−	−	−	−	+	−
	VH	+	−	−	−	−	−	−	−	−	−	+	−
	MY	−	−	−	−	−	−	−	+	−	−	−	−
	HO	−	−	−	−	−	−	−	−	−	+	+	+
	WA	+	−	+	+	+	−	+	+	+	+	+	+
	MZ	+	−	−	−	+	+	+	+	+	+	+	+
III	H	−	−	−	−	−	−	−	−	+	−	+	−
	BE	−	−	−	−	−	−	+	−	−	−	−	−
	MA	−	−	−	+	−	−	−	−	−	−	−	−
	WI	+	−	−	−	−	+	−	−	+	−	+	−
	SE	+	+	+	+	+	+	+	+	+	+	+	+
	LA	+	+	+	+	−	+	−	+	+	+	+	−
	T	+	+	+	+	+	+	+	+	+	+	+	+

[a] +, Opsonic activity present; −, opsonic activity absent.

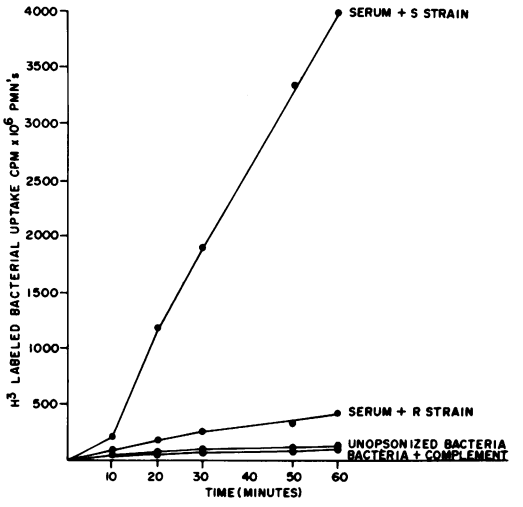

FIG. 1. *^{3}H-radiolabeled bacterial uptake of type III group B streptococci by serum with opsonins to the serum-sensitive (S) strain and not to the serum-resistant (R) strain.*

TABLE 3. *Production of type-specific polysaccharide antigen by resistant and sensitive type II and III group B streptococci*

Type	Strain isolate	Serum sensitivity	Dilution of extract[a] reacting with typing serum	
			CIE	Capillary precipitins
II	WA	S	1:100	1:32
	HAR	S		1:64
	HO	R	1:50	1:16
	GA	R		1:32
III	T	S	1:50	1:16
	SE	S		1:32
	D	S		1:16
	H	R	1:100	1:64
	LY	R		1:64

[a] After incubation for 10 to 12 h in enriched, buffered Todd-Hewitt medium, the culture was adjusted to colony count of 10^9/ml, and the extracts were made from equal volumes. CIE, Counterimmunoelectrophoresis.

nisms. To better delineate the factors responsible for strain variation in opsonic activity, a number of absorption studies were carried out comparing resistant and sensitive strains of the same type. When serum contained antibody (opsonic and indirect immunofluorescent) against the S strain but lacked antibody to the R strain, absorption with whole cells of that S strain completely removed the opsonic activity. In contrast, absorption with an R strain removed some but not all of the opsonins to the S isolate (Fig. 2). This suggests that the R and S isolates have at least one common antiphagocytic factor.

When serum contained antibody to both the S and R strains, the S strain was opsonized with R strain-absorbed serum but not with S strain-absorbed serum (Fig. 3). Absorption with either the R or the S strain of the same type removed all of the opsonic activity to the R isolate. The R strain must, therefore, require antibody to both the common factor and to an added antiphagocytic factor for its opsonization.

Further absorption studies with several sensitive strains of the same type revealed additional strain-specific differences in opsonic activity. Absorption of serum with antibody to one S strain with a second S strain to which it contained antibody only partially decreased the op-

sonic activity to the first strain (Fig. 4). These results suggest that the S strains share a common antigen but also have a strain-specific antigen.

Absorption studies with extracts of organisms. To further characterize the antiphagocytic factors of the group B streptococcal strains, absorption studies were carried out with buffered saline, hydrochloric acid, and trichloroacetic acid extracts of whole organisms. These extracts contain the capsular polysaccharide antigens of the group B streptococcus (11-14).

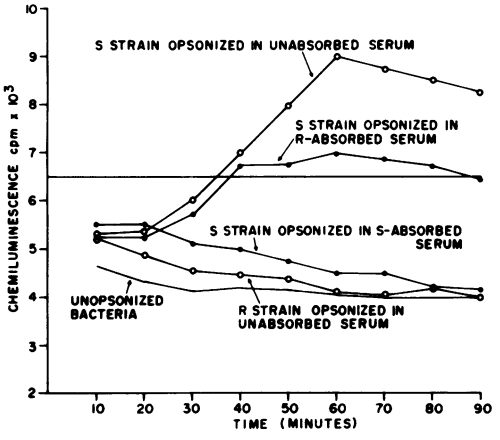

FIG. 2. *Absorption studies with whole type III group B streptococci by the CL technique. Serum contained opsonins to the S strain but lacked opsonins to the R strain.* (○) *Unabsorbed serum;* (●) *serum absorbed with whole bacteria.*

These extracts removed a portion of the opsonic activity directed against the S strain and all of the activity for the R strain (Fig. 5). It appears, therefore, that the common antigen for S and R strains is present in these extracts.

Modification of group B streptococcal surface antigens. Attempts were made to remove an antigen from resistant or sensitive bacteria by trypsin or neuraminidase treatment. Trypsin was used because protein antigens have been identified to which mouse protective antibody is formed in rabbits (17). Neuraminidase potentially might degrade portions of the sialic acid-containing capsular polysaccharide antigen. When serum contained antibody to both the sensitive and resistant organisms, higher CL levels were induced for both sensitive and resistant group B streptococci by trypsin treatment (Fig. 6). These results suggest that cell surface proteins on these organisms may have antiphagocytic properties. Neuraminidase treatment was not found to affect opsonization of these group B streptococci, whether bacteria were grown in media containing neuraminidase or were treated subsequently for 3 h at 37°C. This result most likely reflects an inability of the neuraminidase to significantly degrade this sialic acid-containing antigen (13, 17).

Protein antigens identified by polyacrylamide gel electrophoresis. Preliminary attempts to further define the mechanism of action of trypsin on these bacteria utilized polyacrylamide gel electrophoresis. A qualitative difference in protein among type II- and type III-resistant and -sensitive strains (12 strains ex-

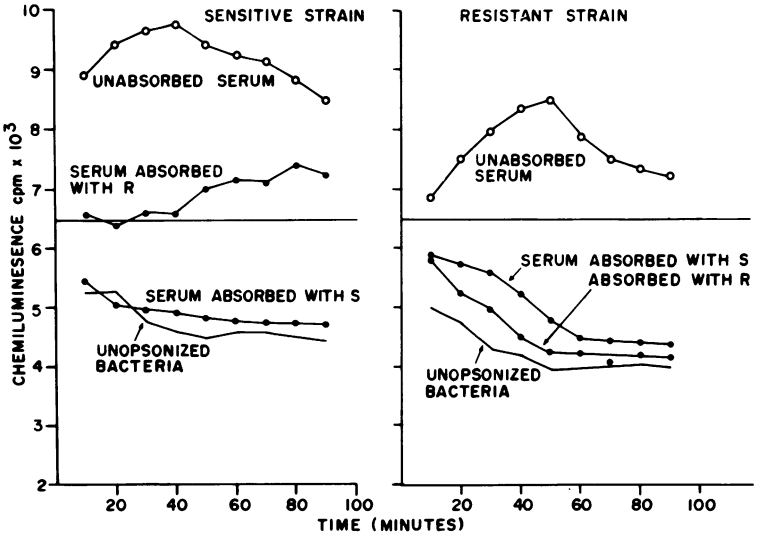

FIG. 3. *Absorption studies with whole type II group B streptococci. Serum contained opsonins to both S and R strains.* (○) *Unabsorbed serum;* (●) *absorbed serum.*

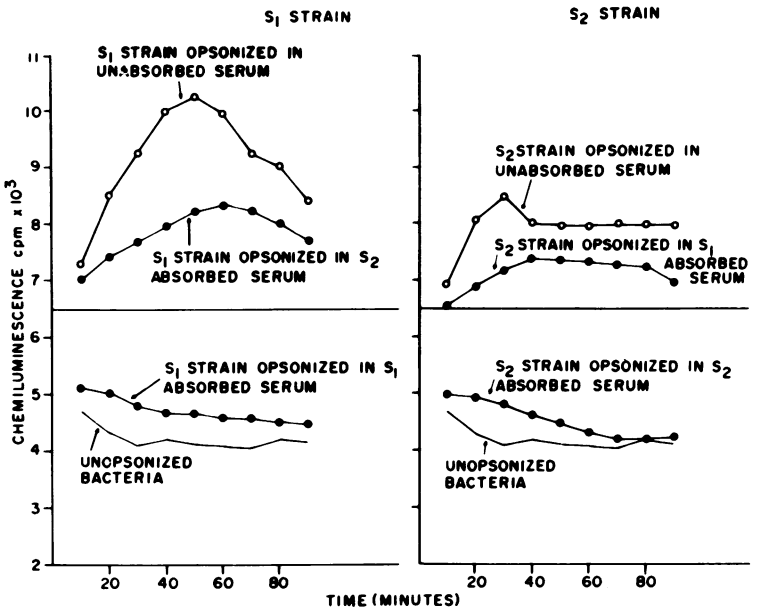

FIG. 4. *Absorption studies with whole type III group B streptococci. Serum contained opsonins to both S strains.* (○) *Unabsorbed serum;* (●) *absorbed serum.*

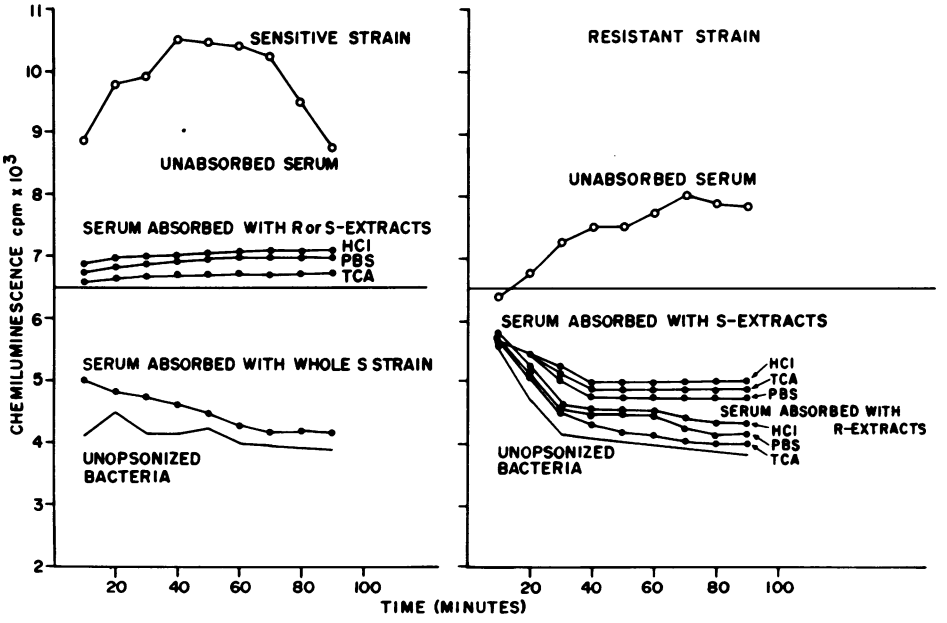

FIG. 5. *Absorption studies with HCl, trichloroacetic acid, or saline extracts of type II group B streptococci. Serum contained opsonins to both S and R strains.* (○) *Unabsorbed serum;* (●) *serum absorbed with a bacterial extract.*

amined) has not been observed. Further attempts to define a qualitative protein difference by trypsinization and autoradiography are in progress.

DISCUSSION

Our results suggest that the opsonic antigen-antibody systems of group B streptococci types II and III are somewhat more complicated than

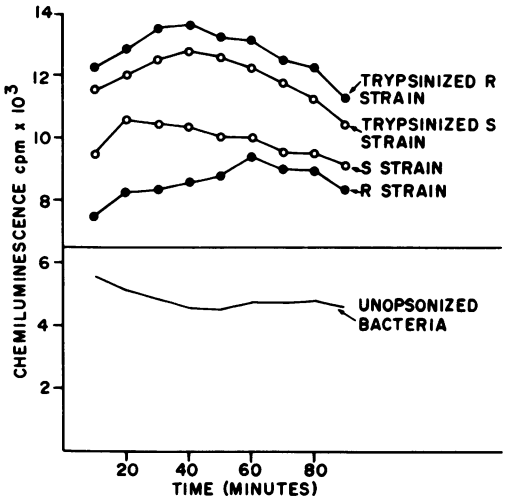

FIG. 6. *Effect of trypsin treatment on opsonization of type II group B streptococci. Serum contained opsonins to both S and R strains.* (○) *S strain;* (●) *R strain.*

is generally appreciated. Type-specific antibody prevalences for the reference strains in normal adult sera are approximately 40% for type II and approximately 75% for type III (9). The present study indicates, however, that strains of type II and III group B streptococci are not uniformly susceptible to opsonization by human sera containing type-specific antibody. In these studies CL levels have correlated well with phagocytosis by visual microscopic examination of reaction mixtures, and deposition of immunoglobulin G has been demonstrated on the bacterial surface by indirect immunofluorescence. In addition, studies of phagocytosis utilizing a radiolabeled group B streptococcal technique confirmed that serum opsonic for one group B streptococcal strain did not opsonize all other strains of the same type.

One factor responsible for the difference might be the quantity of type-specific capsular polysaccharide antigen present on each strain. *Streptococcus pneumoniae* with thicker capsules are more virulent, appear to elicit lower antibody levels, and are less readily opsonized in human sera (20). However, opsonization of pneumococci via the alternative pathway of complement does not appear to be dependent on capsular polysaccharide (7, 26). The virulence of *Haemophilus influenzae* type b appears to be associated with the amount of physically integrated capsular polysaccharide, which increases the resistance to bloodstream clearance (24). Strains of group B streptococci are known to vary in capsular polysaccharide amounts, and in some instances are missing this antigen (18, 22). The addition of unincorporated homologous capsular polysaccharide obtained from trichloroacetic acid extracts has also been reported to inhibit opsonic activity in an in vitro opsonophagocytic assay (4). Group B streptococci grown in an enriched Todd-Hewitt medium have been reported to have increased capsular polysaccharide antigen present on the bacterial surface (3). However, increased polysaccharide incorporated on live group B streptococci has not been studied for increased virulence in the mouse protection system or for resistance to bactericidal killing by in vitro assays. We could not, however, significantly affect opsonization of a strain by use of enriched media. Attempts to quantitate extracts of the capsular polysaccharide by dilutions in capillary precipitin and counterimmunoelectrophoresis studies failed to show a correlation of sensitive or resistant strains with capsular antigen quantities.

A number of antigens have been identified for group B streptococci. These include the group B antigen, a polysaccharide that is not associated with virulence, opsonic antibody, or mouse protection (9, 18). Type-specific polysaccharide antigens include (i) Ia, common for types IA and Ic; (ii) Ib, present on Ib; (iii) Iabc, common for Ia, Ib, and Ic; (iv) II, identifying II; and (v) III, identifying III. These type-specific polysaccharides contain sialic acid. A protein antigen Ibc is found on types Ib and Ic. In addition, proteins called X and Y have been identified (22). Antibodies in rabbit hyperimmune sera to both polysaccharide (Ia, II, III) and protein antigens (Ibc) have been found to confer mouse protection (19). In addition, the presence of cross-reacting heat-stable opsonins to type Ic by antisera to type III organisms (1) and to type III by antisera to type Ia (4) has been suggested in studies utilizing rabbit hyperimmune antisera. Although the Ibc protein is occasionally found in type II and type III strains, antibody to this antigen did not appear to be responsible for opsonic activity (1). Studies of human sera have indicated a major role for antibody to type-specific capsular polysaccharide, but a role for antibody to other antigens has not been identified. Our previous studies using the CL assay showed that absorption with trichloroacetic acid and hot HCl extracts containing the type-specific capsular polysaccharide removed some but not all opsonins to these organisms (9).

The present studies indicate that the antibody to type-specific capsular polysaccharide is important in opsonizing both resistant and sensitive strains. In addition, however, antibody to a strain-specific factor is capable of opsonizing sensitive group B streptococci. Resistant organisms appear to require antibody to both the

type-specific capsular polysaccharide antigen and to an added antiphagocytic factor. Since trypsin treatment produced significantly enhanced opsonization of resistant and sensitive strains, proteins present on some group B streptocci may be important antiphagocytic factors. Polyacrylamide gel electrophoresis studies to define protein differences are currently under way in our laboratory. Since one protein antigen (28R) found on some group A, B, C, and G streptococci has not been associated with more protection, our findings require further investigation to determine the in vivo significance of these antiphagocytic factors (17).

Our results suggest that type-specific opsonins alone may not protect against infection with some strains of group B streptococci. Therapy with gamma globulin or plasma preparations may require selection of donors with high antibody titers to resistant organisms. In addition, development of efficacious vaccines will require consideration and study of these strain-specific differences.

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