THE ROLE OF SPECIFIC ANTIBODY IN ALTERNATIVE COMPLEMENT PATHWAY-MEDIATED OPSONOPHAGOCYTOSIS OF TYPE III, GROUP B *STREPTOCOCCUS**

By MORVEN S. EDWARDS,† ANNE NICHOLSON-WELLER, CAROL J. BAKER,§ AND DENNIS L. KASPER‖

From the Channing Laboratory, the Thorndike Laboratories, and the Departments of Medicine, Robert B. Brigham and Peter Bent Brigham Hospitals, Division of Affiliated Hospital Centers, Inc.; and Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts and the Departments of Pediatrics, and Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

One surface moiety that has been found to modulate activation by the alternative pathway is sialic acid. Activating particles such as zymosan and rabbit erythrocytes are relatively deficient in surface-associated sialic acid, whereas nonactivating particles such as sheep erythrocytes contain an abundance of sialic acid moieties (1–3). Enzymatic removal of sialic acid residues with neuraminidase (1, 2) or conversion by mild oxidation with sodium periodate and reduction with borohydride to heptulosonic acid (1) converts the sheep erythrocyte from a nonactivating to an activating surface for the alternative pathway. Activation by sheep erythrocytes requires removal or modification of at least 40% of membrane sialic acid, and increases proportionately when larger amounts are affected (1). Surface-associated sialic acid modulates alternative pathway function by increasing the affinity of β1H relative to B for C3b. This results in blocking formation of the alternative pathway C3 convertase C3bBb (1–3).

The native polysaccharide antigen of type III, group B *Streptococcus* consists of a repeating unit of galactose, glucose, glucosamine, and α-D-*N*-acetylneuraminic acid in a molar ratio of 2:1:1:1 (4, 5). The native antigen isolated from organisms by gentle extraction procedures contains one sialic acid residue for each repeating unit, and these residues completely mask all the end group galactopyranose units in the antigen (5). If one could extrapolate the sialic acid-rich capsule of type III from mammalian erythrocytes, group B streptococci might be expected to function as a nonactivating surface for the alternative complement pathway.

Several studies have failed to define a significant role for the alternative pathway in opsonophagocytosis of group B streptococci (6–8). These studies have employed sera with little opsonic activity as assessed by chemiluminescence for the test bacterium. A crucial role for type-specific antibody in host defense against group B streptococcal infection has been defined (9, 10), but the role of type-specific antibody

* Supported by grant AI 13249 and contract AI 72538 from the National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Md. Presented in part at the Society for Pediatric Research Meeting, Atlanta, Ga., May 1979.

‡ Recipient of NIAID Research Fellowship Award F32 AI05841.

§ Recipient of NIAID Research Career Development Award 1 K04 AI00323.

‖ Recipient of NIAID Research Career Development Award 1 K04 AI00126.

in alternative pathway-mediated bactericidal activity for type III, group B *Streptococcus* has not been investigated. Although efficient activators of the alternative pathway such as zymosan (11) and rabbit erythrocytes (12, 13) require only C3, B, $\bar{D}$, properdin, β1H, and the C3b inactivator (C3BINA) for cleavage of C3, immunoglobulin can have a facilitatory role (14, 15). For example, IgG participates in the alternative pathway-mediated opsonization of *Streptococcus pneumoniae* (16, 17), *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* (18), *Shigella* (19), *Pseudomonas aeurginosa* (20, 21), group C meningococcus (22), and some strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* (23).

These studies were designed to define the participation of the alternative complement pathway in opsonophagocytosis of type III, group B *Streptococcus* and the role of specific antibody directed to the sialic acid-rich native capsular polysaccharide antigen in facilitating alternative pathway activation.

Materials and Methods

Bacteria. Strain M732 of type III, group B *Streptococcus*, was isolated from the cerebrospinal fluid of a neonate with meningitis. Organisms were stored at −70°C in 0.5-ml aliquots of Todd-Hewitt Broth (THB)[1] (Difco Laboratories, Detroit, Mich.). Frozen organisms were thawed and streaked onto a blood-agar plate and incubated overnight at 37°C. Two loopfuls of plate-grown organisms were inoculated into 250 ml nephelometry flasks containing 30 ml of THB and grown at 37°C for ~2 h to an OD of 0.1 (starting OD = .02) at 650 nm as determined by a Coleman Junior Spectrophotometer (model 6C, Coleman Systems, Irvine, Calif.). Cultures grown in this manner are in early log-phase growth (24). 30 ml of broth culture was centrifuged at 5,000 rpm for 10 min, and the pellet was resuspended in 16 ml of minimal essential medium (MEM) with Earle's balanced salt solution (Microbiological Associates, Walkersville, Md.). A final 1/10 dilution of the bacterial suspension in MEM was required to achieve a ratio of bacteria:leukocytes (WBC) of ~3–4:1 in the opsonic reaction mixture.

Neuraminidase-treated Bacteria. Enzymatic removal of sialic acid residues was accomplished by incubating plate-grown organisms in glucose-enriched, non-pH-titrated THB (25) containing 0.43 U/ml of *Clostridium perfringens* neuraminidase (type V, lot 118 C-8020; Sigma Chemical Co., St. Louis, Mo.). 1 U of *C. perfringens* neuraminidase liberates 1 μmol of *N*-acetyl neuraminic acid per min from *N*-acetyl neuramin-lactose at pH 5.0 at 37°C. The growth medium was supplemented once during an incubation period of ~3 h with neuraminidase (0.43 U/ml THB) and organisms were harvested when the pH reached 5.0. Cultures grown in this manner are in mid-log-phase growth (24, 25). After washing in THB, the pellet was resuspended to an OD of 0.1 at 650 nm and diluted as described for the opsonic assay. Control organisms were incubated in the same manner in glucose-enriched THB titrated using 1 N NaOH to maintain the pH at 7.0–7.5.

Preparation of Rabbit Antisera. New Zealand White rabbits were immunized with formalin-killed type III, group B streptococci grown in pH-titrated THB according to the method of McCarty and Lancefield (26). Hyperimmune rabbit antiserum to *Streptococcus pneumoniae* type 14 was prepared in the same manner.

Human Sera. Aliquots of human sera were frozen at −70°C within 1 h after collection to preserve complement activity. Genetically C2-deficient (C2D) human serum was obtained from a woman with inactive systemic lupus erythematosis who had normal levels of C3, B, C3-inactivator, and β1H (27). Agammaglobulinemic serum was obtained from a child with severe combined immunodeficiency (IgG: 14 mg/100 ml, IgA and IgM: undetectable) and normal complement levels except for C1q which was 30% of the normal serum pool value (28).

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; C3BINA, C3B inactivator; C2D, C2 deficient; CFU, colony-forming units; GVB, isotonic, pH 7.5, veronal-NaCl buffer that contained 0.1% gelatin; GVB^{++}, GVB with 0.15 mM Ca^{++} and 0.5 mM Mg^{++}; MEM, minimum essential medium; MgEGTA, GVB supplemented to 4 mM Mg^{++} and 16 mM EGTA and adjusted to pH 7.5; THB, Todd-Hewitt broth; WBC, leukocytes.

Complement activity in selected sera was inactivated by heating to 56°C for 30 min. Concentrations of type III, group B streptococcal antibody were determined with a radioactive antigen-binding assay described previously (10). The sera used as sources of antibody contained concentrations of antibody directed against the capsular polysaccharide antigen of the test bacterium that are representative of the natural range found in normal adults. In this opsonophagocytic assay, >90% reduction in colony-forming units (CFU) has been uniformly observed with adult sera that contained >2 μg/ml of type-specific antibody. The majority of adult sera that contained <2 μg/ml of antibody have failed to kill 90% of the initial inoculum (29).

Buffers. Isotonic, pH 7.5, veronal-NaCl buffer, that contained 0.1% gelatin (GVB) made to 0.15 mM Ca^{++} and 0.5 mM Mg^{++} (GVB^{++}) was prepared as described (30). MgEGTA buffer was made by supplementing GVB to 4 mM Mg^{++}, and 16 mM EGTA and adjusting to pH 7.5. Dilution of serum 1/2 with MgEGTA to chelate calcium permitted C3 activation by zymosan (85% consumption), but not by antibody-coated sheep erythrocytes.

Complement Components. Functionally purified guinea pig C2 was prepared by the method of Borsos et al. (31).

Preparation of Leukocyte Suspension. 20 ml of peripheral venous blood was collected from normal volunteers in a nonheparinized plastic syringe. 3-ml aliquots of blood were added to 12- × 75-mm polypropylene tubes (Fisher Scientific Co., Pittsburgh, Pa.) each containing 1 ml 6% dextran (Dextran T70, Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) and 0.75 ml citrate solution (16 g citric acid and 50 g sodium citrate per liter). The erythrocytes were sedimented at 37°C for 45 min. The WBC-rich plasma supernate was separated and washed twice in MEM with 1% bovine serum albumin (BSA), (Armour Pharmaceutical Co., Chicago, Ill.) before and after lysis of contaminating erythrocytes with 0.84% ammonium chloride. The WBC were resuspended in MEM with 1% BSA at 1×10^7 WBC/ml.

Opsonophagocytic Assay. A modification of the opsonophagocytic assay of Baltimore et al. (24) was employed. 12- × 75-mm polypropylene tubes contained a total reaction mixture vol of 0.4 ml that consisted of 0.1 ml WBC suspension ($\sim 1 \times 10^6$ WBC), 0.1 ml bacterial suspension ($\sim 3 \times 10^6$ CFU), 0.1 ml of serum as an antibody source and 0.1 ml of fresh serum. When exogenous complement was not employed, 0.1 ml of MEM was substituted. Control tubes lacking either WBC, or test serum, or complement were included in each experiment. Tubes were incubated for 40 min at 37°C with end-over-end rotation in a Fisher Roto-Rack apparatus.

Pre- and postincubation specimens (25 μl) were removed and WBC lysed in sterile distilled water (1/10 dilution). Four additional 1/10 dilutions in 0.15 M NaCl were prepared. A 10-μl specimen from each of the four final dilutions was streaked on a blood-agar plate which was incubated overnight at 37°C. Colonies were counted using a Quebec Colony Counter (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). Results were expressed as the percent decrease of the initial number of viable bacteria as:

$$100 - \left[\frac{\text{CFU at 40 min}}{\text{CFU at 0 min}} \times 100\right].$$

Results represent the mean of a minimum of two determinations.

When the classical pathway was inhibited by MgEGTA, the experimental procedure was modified to include a preopsonization step to avoid toxicity to the WBC. Sera diluted 1/2 in MgEGTA or GVB^{++} as a control were incubated 5 min at 37°C to allow the EGTA to chelate the available calcium. After the addition of bacteria (0.1 ml of 3×10^7 CFU/ml) the mixtures were opsonized for 30 min at 37°C with end-over-end rotation, and then chilled and centrifuged. The bacteria were washed once in MEM and then resuspended in MEM with or without added WBC for continuation of the phagocytic-killing assay as defined above.

Absorption of Sera. 2-ml aliquots of heat-inactivated sera were absorbed using a pellet of washed, whole, early-log-phase type III, group B streptococci or purified type III native capsular polysaccharide antigen (4) coupled to sheep erythrocytes by a modification of the method of Baker et al. (32). An absorption control employing purified type 3 pneumococcal polysaccharide antigen (kindly provided by Dr. Richard Markham) was processed in the same manner. 1-ml suspensions of 0.15 M NaCl that contained 2.0 mg of native type III, group B streptococcal polysaccharide or 500 μg of type 3 pneumococcal antigen were added to 0.5-ml pellets of

TABLE I

Demonstration of Alternative Pathway-mediated Bactericidal Activity for Type III, Group B Streptococcus in Serum that Contained Specific Antibody in Sufficient Concentration

Type III-specific antibody concentration of normal human serum used in reaction mixture	Complement source	Bactericidal index*		
		Mean‡	Range	SD
μg/ml				
2.8	Autologous	98	97–98	0.6
0.7	Autologous	82	69–94	8.2
2.8	None	0	-	0
0.7	None	0	-	0
2.8§	C2D	96	94–98	1.3
0.7§	C2D	6	0–12	5.1
0.4	C2D	34	20–46	8.6

* Bactericidal index = $100 - \left[\frac{\text{CFU at 40 min}}{\text{CFU at 0 min}} \times 100\right]$.

‡ Results represent the mean of four experiments.

§ Heat-inactivated at 56°C for 30 min.

packed, washed sheep erythrocytes. Preliminary studies had shown these concentrations were optimal for sensitization of the sheep erythrocytes with each of these antigens. 1 ml of 0.1% chromium chloride ($CrCl_3 \cdot 6\ H_2O$) was added and the mixture allowed to stand at 25°C for 5 min. After washing the antigen-sensitized sheep erythrocytes with 0.15 M NaCl, 2 ml of heat-inactivated serum was added to each pellet and incubated with shaking for 1 h at 37°C and 1 h at 4°C. After centrifugation at 5,000 rpm for 15 min, the absorbed sera were filtered, aliquoted and stored at −70°C until tested.

Results

Sera containing endogenous complement and moderate (2.8 μg/ml) or low (0.7 μg/ml) concentrations of antibody killed 98 and 82% of the initial bacterial inoculum after a 40-min incubation period (Table I). These values are representative for adult sera previously tested in this system. Heat-inactivated serum failed to opsonize type III, group B streptococci despite moderate levels of antibody. C2D serum that contained low levels of type-specific antibody (0.4 μg/ml) could restore the ability of heated serum that contained 2.8 μg/ml of type-specific antibody to kill these organisms (96% decrease in CFU). C2D serum was not effective in restoring the killing ability of heated serum that contained 0.7 μg/ml antibody (6% decrease in CFU). Reconstitution of C2D serum with 40 U of functionally purified guinea pig C2 restored the bactericidal capacity of C2D serum to 87% reduction in CFU, a level similar to that of normal, antibody-deficient serum. Guinea pig, rather than human C2 was employed, because factor B may be a contaminant of partially purified C2, and heterologous combinations of B are incompatible (33). These results indicate that the killing ability of serum that contained low levels of antibody depends on activation of the classical complement pathway, but with higher levels of antibody, the alternative pathway can be recruited even in the absence of an intact classical pathway.

It was possible that the concentration of antibody was critical in this assay either because of its role in the fixation of complement or because of its interaction with the Fc receptors of the phagocytic cells. The following experiment was designed to

TABLE II

Mechanism of Alternative Pathway-mediated Opsonophagocytosis of Type III, Group B Streptococcus

Type III, group B streptococcal antibody concentration	Complement source	Concentration of type III group B streptococcal antibody added after opsonization procedure	Bactericidal index*
μg/ml		*μg/ml*	
2.8	Autologous	2.8	92
0.7	Autologous	2.8	77
2.8	None	2.8	0
0.7	None	2.8	0
2.8‡	C2D	2.8	94
0.7‡	C2D	2.8	3
0.4	C2D	2.8	0

* Bactericidal index = $100 - \left[\frac{\text{CFU at 40 min}}{\text{CFU at 0 min}} \times 100\right]$.

‡ Heat-inactivated at 56°C for 30 min.

distinguish between these two alternatives. Bacteria were incubated for 30 min with C2D serum and heated serum that contained antibody in moderate (2.8 μg/ml) or low (0.7 μg/ml) concentrations and then washed in buffer. More type-specific antibody (2.8 μg/ml) as contained in heated serum was added to the washed bacteria and finally WBC were added for continuation of the killing assay. The addition of more antibody to bacteria after complement was fixed failed to influence the opsonophagocytic assay (Table II). Therefore, the significance of the critical antibody concentration is in mediating complement activation and not in interacting with the Fc receptors on the phagocytic cells.

Inhibition of the classical pathway by diluting serum 1/2 in MgEGTA buffer during the opsonization step caused no reduction in subsequent phagocytic killing for five sera containing a range from moderate to high concentrations of type-specific antibody (Table III). However, significant inhibition of opsonophagocytosis was observed when the classical complement pathway was inhibited by MgEGTA for five sera containing antibody in very low concentrations ($t = 8.0$, $P < 0.005$). Bacterial growth was observed in reaction mixtures containing MgEGTA or GVB^{++} in the absence of serum.

When bacteria were opsonized for 30 min in sera diluted 1/2 in GVB^{++} before the addition of WBC, both low and moderate to high concentrations of type-specific antibody permitted a large reduction in CFU. Bacteria that have been opsonized before incubation with WBC (Table III) are apparently more readily phagocytized than bacteria simultaneously incubated with serum and WBC (Table I). This increase in killing may be the result of anti-complementary effects of the leukocyte preparation such as nonspecific activation of complement in the fluid phase or the degradation of surface deposited C3b by released proteolytic enzymes from the leukocytes.

The role of antibody to the native capsular polysaccharide antigen in facilitating alternative complement pathway-mediated opsonophagocytosis of type III, group B streptococci was further defined through absorption of sera with type III organisms and purified type III native capsular antigen (4) (Table IV). The initial antibody

TABLE III

Antibody Facilitation of Alternative Pathway-mediated Opsonophagocytosis of Type III, Group B Streptococcus

Serum source	Type III, group B streptococcal antibody concentration		Buffer	Bactericidal index*	
	Mean	Range		Mean	Range
	μg/ml				
Five antibody-deficient sera	0.3	—	GVB^{++}	93‡	85–96
Five antibody-deficient sera	0.3	—	MgEGTA	40‡	9–54
Five sera that contained antibody in moderate to high concentrations	14.9	(2.8–40.2)	GVB^{++}	97	96–98
Five sera that contained antibody in moderate to high concentrations	14.9	(2.8–40.2)	MgEGTA	97	95–98

* Bactericidal index = $100 - \left[\frac{\text{CFU at 40 min}}{\text{CFU at 0 min}} \times 100\right]$.

‡ Difference between bactericidal index of antibody-deficient sera in GVB^{++} vs. MgEGTA buffers is significant by paired t test ($t = 8.0$, $P < 0.005$).

TABLE IV

Role of Antibody to the Native Capsular Polysaccharide Antigen in Alternative Pathway-mediated Opsonophagocytosis of Type III, Group B Streptococcus

Serum	Absorption	Final type III GBS antibody concentration	Complement	Bactericidal index*
		μg/ml		
NHS_1‡	None	2.8	Autologous	98
ΔNHS_1§	None	2.8	None	0
ΔNHS_1	None	2.8	C2D	90
ΔNHS_1	Type III GBS cells	0.3	None	0
ΔNHS_1	Type III GBS polysaccharide	0.4	None	0
ΔNHS_1	Type III GBS cells	0.3	C2D	0
ΔNHS_1	Type III GBS polysaccharide	0.4	C2D	39
ΔNHS_1	Type 3 pneumococcal polysaccharide	2.8	C2D	95
C2D	None	0.4	Autologous	29

* Bactericidal index = $100 - \left[\frac{\text{CFU at 40 min}}{\text{CFU at 0 min}} \times 100\right]$.

‡ NHS_1, Serum from normal donor containing the antibody concentration specified.

§ ΔNHS_1, Normal human serum that was heat-inactivated at 56°C for 30 min.

concentration of 2.8 μg/ml was reduced to 0.3 and 0.4 μg/ml after absorption with whole organisms and native type III polysaccharide antigen, respectively. No reduction in serum antibody concentration occurred after absorption with type 3 pneumococcal polysaccharide antigen. Addition of C2D serum as a complement source did not restore opsonophagocytic activity to serum that had been absorbed with either whole bacteria or purified type III capsular polysaccharide antigen to remove specific antibody. Opsonization was unaffected when specific antibody-containing serum was

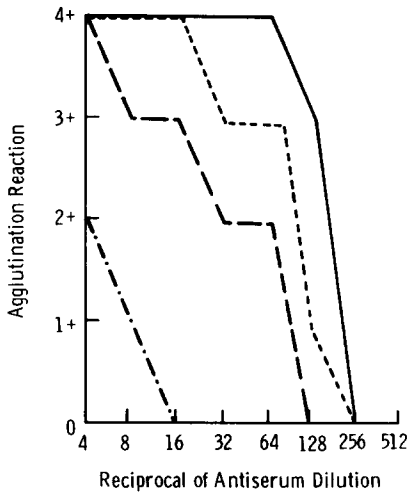

FIG. 1. Slide agglutination reaction graded 4+ (large clumps, clear background), 3+ (large and small clumps, clear background), 2+ (small clumps, milky background), 1+ (fine granularity against a milky background) and negative for: (—) neuraminidase-treated organisms with antiserum to *S. pneumoniae*, type 14; (-·-) pH-titrated organisms with antiserum to *S. pneumoniae*, type 14; (- - -) neuraminidase-treated organisms with antiserum to native type III, group B *Streptococcus*; (----) pH-titrated organisms with antiserum to native type III, group B *Streptococcus*.

preabsorbed with type 3 pneumococcal polysaccharide antigen and then combined with C2D serum as a complement source. These results indicate that sufficient specific antibody directed against the native type III capsular polysaccharide antigen facilitates opsonophagocytosis of type III, group B *Streptococcus* via the alternative complement pathway.

The contribution of terminal sialic acid residues in the type III, group B streptococcal capsule to inhibition of alternative pathway-mediated opsonophagocytosis by sera deficient in type-specific antibody was determined by experiments in which the immunologic specificity of the organisms was modified by neuraminidase cleavage of the terminal sialyl residues. Removal of the sialic acid results in an antigen identical to the capsular polysaccharide of *S. pneumoniae*, type 14 (5, 34). Organisms grown in the presence of *C. perfringens* neuraminidase in an acidic medium exhibited strong agglutination reactions with antiserum to *S. pneumoniae*, type 14 and weaker agglutination reactions with antiserum to the native type III, group B streptococcal antigen, prepared using organisms grown at neutral pH to prevent cleavage of the acid-labile sialic acid end-groups (4, 34) (Fig. 1). Control non-neuraminidase-treated organisms grown in broth titrated to maintain a neutral pH exhibited strong agglutination reactions with antiserum to the native antigen and agglutinated minimally with antiserum to *S. pneumoniae*, type 14.

The inhibition of bactericidal activity previously observed when sera with low concentrations of type-specific antibody were opsonized in MgEGTA buffer was overcome when neuraminidase-cleaved organisms were added to the subsequent phagocytic mixture (Fig. 2). The alternative pathway-mediated bactericidal capacity of antibody-deficient sera for the partially desialated type III, group B streptococci was equal to that of sera that contained specific antibody in higher concentrations.

Because cleavage of all terminal sialic acid residues yields an incomplete type III

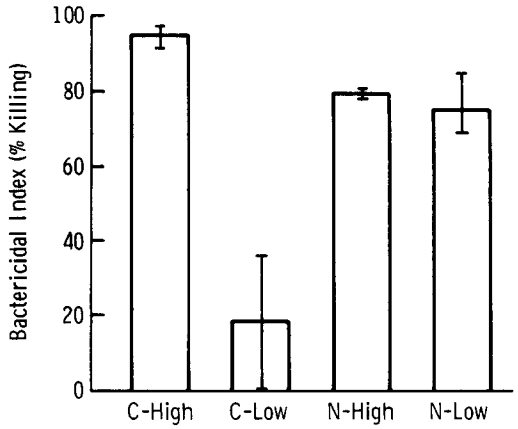

FIG. 2. Bactericidal index for alternative pathway-mediated opsonophagocytosis of type III, group B streptococci grown in pH-titrated media (C) or in the presence of neuraminidase (N) by sera that contained high or low concentrations of type-specific antibody. Mean and range in bactericidal indices are indicated for four sera.

capsular antigen which is structurally identical to the *S. pneumoniae*, type 14 polysaccharide antigen (5, 34), it was possible that antibody directed to the partially unmasked galactopyranose residue was mediating opsonophagocytosis of the enzymatically modified bacteria. Agammaglobulinemic serum was employed to determine whether bactericidal activity for the cleaved organisms was mediated by antibody directed to an immunodeterminant exposed by neuraminidase treatment or if partial desialation had converted the enzyme-treated organisms into non-antibody-dependent activating particles for the alternative pathway. Agammaglobulinemic serum contained no detectable antibody to the *S. pneumoniae*, type 14 or the native type III, group B streptococcal antigens. 97% reduction in the inoculum of neuraminidase-treated bacteria occurred in MgEGTA buffer, whereas no killing of fully sialated organisms was observed in this buffer system. These results demonstrate that alternative pathway-mediated bactericidal activity for partially desialated type III, group B streptococci is not antibody dependent.

Discussion

The preceding experiments show that complement is required for the opsonophagocytosis of type III, group B *Streptococcus*. The effect of a critical concentration of antibody in promoting opsonophagocytosis was mediated by complement fixation and not by interaction of antibody with neutrophil Fc receptors because additional antibody after complement was fixed failed to influence opsonophagocytosis (Table II). Low levels of antibody utilize the classical pathway, but higher levels of specific antibody, in the range correlated with in vivo protection in infants (9), recruit the alternative pathway. Three possible mechanisms may be involved in antibody-dependent activation of the alternative pathway.

First, the role of immunoglobulin in the recruitment of the alternative pathway might depend on deposition of C3b (35, 36) by the classic complement pathway 142 convertase. C3b, the major cleavage fragment of C3, participates in the assembly of the alternative complement C3 cleaving convertase (37). The studies using C2D serum

demonstrate that the classic pathway is not needed to recruit the alternative pathway for opsonization of group B streptococci when there is sufficient antibody.

Secondly, May and Frank (38, 39) described a C1-bypass mechanism of complement activation. The pathway depends on high concentrations of specific antibody, C1 and B for activation of C3. In our experiments, MgEGTA was used to dilute serum for chelation of Ca^{++} and inactivation of C1. The results show that MgEGTA-diluted serum was comparable to normal serum and C2D serum in supplying a complement source. These results rule out a critical role for the C1 bypass mechanism for the antibody-dependent activation of the alternative pathway for opsonization of group B streptococci.

Finally, the surface of an alternative pathway activator must capture the C3b generated by the Mg^{++}-dependent fluid-phase C3 convertase formed by the interaction of native C3, B, properdin, and $\bar{D}$ in the presence of β1H and C3bINA (11–13). The enzymatic activity of this convertase is held in check by β1H which accelerates the intrinsic decay of C3bBb (40, 41) and augments the activity of the C3bINA in irreversibly destroying the binding site for B (42). Sialic acid is one membrane constituent that has been shown to modulate surface activation of the alternative pathway (1, 2). Surfaces of activating particles for the alternative pathway have little sialic acid, whereas nonactivating surfaces are relatively rich in sialic acid. Surface-associated sialic acid greatly increases the binding affinity of β1H for C3b, thereby accelerating intrinsic decay of the convertase, whereas the decreased affinity of β1H for the surface of an activating particle favors formation of the convertase on bound C3b (1–3).

The native polysaccharide antigen of type III, group B *Streptococcus* contains an abundance of sialic acid residues that are all present as endgroups, completely masking the peripheral galactopyranosyl residues of the repeating unit of the antigen (5, 34). Our experiments indicate that this particle functions as a nonactivating surface for the alternative pathway in sera that contained low concentrations of specific antibody. However, in the presence of a critical concentration of antibody with specificity for the sialic acid-containing immunodeterminant, alternative pathway activation occurs. The specificity of this antibody requirement was demonstrated by the complete inhibition of alternative pathway-mediated opsonization after absorption of serum that contained 2.8 μg/ml of type-specific antibody with either whole type III organisms or native type III capsular polysaccharide.

The role of terminal sialyl residues in creating an antibody-dependent requirement for activation of the alternative pathway was then explored. Because enzymatic removal of sialic acid residues on sheep erythrocytes will convert this particle from a nonactivating to an activating surface for the alternative pathway (1), type III, group B streptococcal terminal sialic acid residues were enzymatically cleaved to examine the effect that altering this surface moiety would have on the requirement for specific antibody. Bacteria grown in the presence of neuraminidase exhibited reduced agglutination with antibody to the native capsular antigen but reacted strongly with antibody to *S. pneumoniae*, type 14. Because the nonsialated core antigen of type III, group B *Streptococcus* is structurally identical to the *S. pneumoniae*, type 14 antigen (5, 34), these serologic reactions indicated that neuraminidase had cleaved a portion of the terminal sialic acid residues. Antibody-deficient sera exhibited alternative pathway-mediated bactericidal activity for the partially desialated organisms that was

equal to that observed in sera with antibody concentrations sufficient for alternative pathway activation. This ruled out the possibility that antibody directed against unmasked galactopyranose residues was mediating alternative pathway activation. Therefore, once sialic acid is removed antibody is no longer required for activation of this pathway.

These experiments do not elucidate the mechanism by which antibody directed at an immunodeterminant that contains a terminal sialic acid residue permits alternative pathway activation. One possibility is that the antibody binds and neutralizes the sialic acid residues resulting in impaired β1H binding to C3b, which would allow the formation of a deregulated C3bBb convertase (1). Alternatively, carbohydrate groups on the immunoglobulin might provide a protected site for C3b deposition that is topically removed from the bacterial capsular sialic acid residues.

A receptor for activators of the alternative pathway that permits ingestion in the absence of exogenous proteins has been demonstrated for human blood monocytes (43, 44). The possibility that specific antibody or neuraminidase cleavage created an activating particle that could be directly ingested by monocytes in the opsonic reaction mixture was ruled out by including a serum-free control in each opsonophagocytosis test. Because bacterial growth was always observed, these findings confirmed the complement dependence of bactericidal activity and excluded a significant role for leukocyte-mediated opsonization in the absence of exogenous proteins in this assay system.

These experiments have shown an essential role for complement in this opsonophagocytosis assay. The essential ligand is presumably C3b which is the most important opsonic ligand generated during complement activation (45). Potentially, fixation of C3b by the alternative pathway is a major factor in natural host defense because activation by this pathway can occur in the absence of antibody (11–13), whereas activation of the classical complement pathway requires specific antibody (46). Our experiments show that it is sialic acid residues on the type III, group B streptococcal capsule that allow this pathogen to evade this natural host-defense mechanism. Only the presence of specific anti-capsular antibody, i.e., acquired immunity, will permit alternative complement pathway activation by the organism in its fully sialated state.

It is of interest that several virulent bacteria other than group B *Streptococcus* have sialic acid residues in their capsules. The type III, group B *Streptococcus* presents itself to the host as a fully sialated surface as do serogroups B and C *Neisseria meningitidis* and K1 *Escherichia coli* (47, 48). Interestingly, less-pathogenic serogroups of meningococci and *E. coli* that have some sialic acid in their capsules have this present in a repeating unit, but it is not the exclusive terminal sugar.

Summary

The native capsular polysaccharide antigen of type III, group B *Streptococcus* contains a terminal sialic acid residue on each repeating unit that masks all end-group galactopyranose residues and prevents alternative pathway complement activation by adult human sera in the absence of type-specific antibody. The critical role of the sialic acid residues in allowing the organism to evade activating the alternative complement pathway was shown when neuraminidase treatment of the organism converted the bacteria to activators of the alternative pathway as assessed in agam-

maglobulinemic serum. The requirement for specific antibody in permitting alternative pathway activation by the fully sialated bacteria was shown when sera that contained low levels of specific antibody failed to activate this pathway, and when prior absorption of serum that contained higher type-specific antibody levels with the capsular antigen failed to activate this pathway. The use of C2-deficient sera showed that the classical pathway was not required for antibody-dependent alternative pathway activation. The use of isotonic, pH 7.5, veronal-NaCl buffer that contained 1% gelatin and that was supplemented to 4 mM Mg^{++} and 16 mM EGTA and adjusted to pH 7.5 (MgEGTA) ruled out the participation of the C1-bypass pathway. The presence of sialic acid on the bacterial surface is one means of evading an important mechanism of natural immunity, namely activation of complement by the alternative pathway. Only specific antibody, i.e., acquired immunity, can overcome this virulence factor.

The authors greatly appreciate the excellent secretarial assistance of Jo Ann Bynes and the technical assistance of Bette J. Webb.

Received for publication 15 January 1980.

References

1. Fearon, D. T. 1978. Regulation by membrane sialic acid of β1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. *Proc. Natl. Acad. Sci. U. S. A.* **75:**1971.
2. Pangburn, M. K., and H. J. Müller-Eberhard. 1978. Complement C3 convertase: cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. *Proc. Natl. Acad. Sci. U. S. A.* **75:**2416.
3. Kazatchkine, M. D., D. T. Fearon, and K. F. Austen. 1979. Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and β1H for cell-bound C3b. *J. Immunol.* **122:**75.
4. Baker, C. J., D. L. Kasper, and C. E. Davis. 1976. Immunochemical characterization of the "native" type III polysaccharide of group B *Streptococcus*. *J. Exp. Med.* **143:**258.
5. Jennings, H. J., K. G. Rosell, and D. L. Kasper. 1980. Structural determination and serology of the native polysaccharide antigen of type III, group B *Streptococcus*. *Can. J. Biochem.* **58:**112.
6. Shigeoka, A. O., R. T. Hall, V. G. Hemming, C. D. Allred, and H. R. Hill. 1978. Role of antibody and complement in opsonization of group B streptococci. *Infect. Immun.* **21:**34.
7. Hemming, V. G., R. T. Hall, P. G. Rhodes, A. O. Shigeoka, and H. R. Hill. 1976. Assessment of group B streptococcal opsonins in human and rabbit serum by neutrophil chemiluminescence. *J. Clin. Invest.* **58:**1379.
8. Hill, H. R., A. O. Shigeoka, R. T. Hall, and V. G. Hemming. 1979. Neonatal cellular and humoral immunity to group B streptococci. *Pediatrics.* **64**(Suppl.)**:**787.
9. Baker, C. J., D. L. Kasper, I. B. Tager, A. Paredes, S. Alpert, W. M. McCormack, and D. Goroff. 1977. Quantitative determination of antibody to capsular polysaccharide in infection with type III strains of group B *Streptococcus*. *J. Clin. Invest.* **59:**810.
10. Baker, C. J., and D. L. Kasper. 1976. Correlation of maternal antibody deficiency with susceptibility to neonatal group B streptococcal infection. *N. Engl. J. Med.* **294:**753.
11. Fearon, D. T., and K. F. Austen. 1977. Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **74:**1683.
12. Fearon, D. T., and K. F. Austen. 1977. Activation of the alternative complement pathway

with rabbit erythrocytes by circumvention of the regulatory action of endogenous control proteins. *J. Exp. Med.* **146:**22.

13. Schreiber, R. D., M. K. Pangburn, P. H. Lesavre, and H. J. Müller-Eberhard. 1978. Initiation of the alternative pathway of complement. Recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3948.
14. Polhill, R. B., Jr., S. L. Newman, K. M. Pruitt, and R. B. Johnston, Jr. 1978. Kinetic assessment of alternative complement pathway activity in a hemolytic system. II. Influence of antibody on alternative pathway activation. *J. Immunol.* **121:**371.
15. Nelson, B., and S. Ruddy. 1979. Enhancing role of IgG in lysis of rabbit erythrocytes by the alternative pathway of human complement. *J. Immunol.* **122:**1994.
16. Winkelstein, J. A., H. S. Shin, and W. B. Wood, Jr. 1972. Heat labile opsonins to pneumococcus. III. The participation of immunoglobulin and of the alternative pathway of C3 activation. *J. Immunol.* **108:**1681.
17. Winkelstein, J. A., and H. S. Shin. 1974. The role of immunoglobulin in the interaction of pneumococci and the properdin pathway: evidence for its specificity and lack of requirement for the Fc portion of the molecule. *J. Immunol.* **112:**1635.
18. Bjornson, A. B., and H. S. Bjornson. 1978. Participation of immunoglobulin and the alternative complement pathway in opsonization of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. *J. Infect. Dis.* **138:**351.
19. Reed, W. P., and E. L. Albright. 1974. Serum factors responsible for killing of *Shigella*. *Immunology.* **26:**205.
20. Bjornson, A. B., and J. G. Michael. 1973. Factors in normal human serum that promote bacterial phagocytosis. *J. Infect. Dis.* **128:**S182.
21. Bjornson, A. B., and J. G. Michael. 1974. Factors in human serum promoting phagocytosis of *Pseudomonas aeruginosa*. I. Interaction of opsonins with the bacterium. *J. Infect. Dis.* **130:** S119.
22. Nicholson, A., and I. H. Lepow. 1978. Role of complement in defense against *N. meningitidis*. *Clin. Res.* **26:**525A.
23. Verhoef, J., P. K. Peterson, Y. Kim, L. D. Sabath, and P. G. Quie. 1977. Opsonic requirements for staphylococcal phagocytosis: heterogeneity among strains. *Immunology.* **33:** 191.
24. Baltimore, R. S., D. L. Kasper, C. J. Baker, and D. K. Goroff. 1977. Antigenic specificity of opsonophagocytic antibodies in rabbit anti-sera to group B streptococci. *J. Immunol.* **118:** 673.
25. Baker, C. J., and D. L. Kasper. 1976. Microcapsule of type III strains of group B *Streptococcus*: production and morphology. *Infect. Immun.* **13:**189.
26. McCarty, M., and R. C. Lancefield. 1955. Variation in the group-specific carbohydrate of group A streptococci. *J. Exp. Med.* **102:**11.
27. Nydegger, U. E., D. T. Fearon, and K. F. Austen. 1978. The modulation of the alternative pathway of complement in C2-deficient human serum by changes in concentration of the component and control proteins. *J. Immunol.* **120:**1404.
28. South, M. A., J. R. Montgomery, E. Richie, N. Mukhopadhyay, B. S. Criswell, B. F. Mackler, S. R. DeFazio, P. Bealmear, L. R. Heim, J. J. Trentin, G. R. Dressman, and P. O'Neill. 1977. Four-year study of a boy with combined immune deficiency maintained in strict reverse isolation from birth. IV. Immunologic studies. *Pediatr. Res.* **11:**71.
29. Edwards, M. S., C. J. Baker, and D. L. Kasper. 1979. Opsonic specificity of human antibody to the type III polysaccharide of group B *Streptococcus*. *J. Infect. Dis.* **140:**1004.
30. Mayer, M. M. 1961. Complement and complement fixation. *In* Experimental Immunochemistry 2nd edition. E. A. Kabat and M. M. Mayer, editors. Chas. C. Thomas, Ft. Lauderdale, Fla. 133.

31. Borsos, T., H. J. Rapp, and C. T. Cook. 1961. Studies on the second component of complement. III. Separation of the second component from guinea pig serum by chromatography on cellulose derivatives. *J. Immunol.* **87:**330.
32. Baker, P. J., P. W. Stashak, and B. Prescott. 1969. Use of erythrocytes sensitized with purified pneumococcal polysaccharides for the assay of antibody and antibody-producing cells. *Appl. Microbiol.* **17:**422.
33. Brade, V., L. Dieminger, G. Schmidt, and W. Vogt. 1976. Incompatibility between C3b and B of guinea-pig and man and its influence on the titration of the alternative pathway factors $\bar{D}$ and B in these two species. *Immunology.* **30:**171.
34. Kasper, D. L., C. J. Baker, R. S. Baltimore, J. H. Crabb, G. Schiffman, and H. J. Jennings. 1979. Immunodeterminant specificity of human immunity to type III, group B *Streptococcus*. *J. Exp. Med.* **149:**327.
35. Root, R. K., L. Ellman, and M. M. Frank. 1972. Bactericidal and opsonic properties of C4-deficient guinea pig serum. *J. Immunol.* **109:**477.
36. Nicholson, A., V. Brade, G. D. Lee, H. S. Shin, and M. M. Mayer. 1974. Kinetic studies of the formation of the properdin system enzymes on zymosan: evidence that nascent C3b controls the rate of assembly. *J. Immunol.* **112:**1115.
37. Müller-Eberhard, H. J., and O. Götze. 1972. C3 proactivator convertase and its mode of action. *J. Exp. Med.* **135:**1003.
38. May, J. E., and M. M. Frank. 1973. Hemolysis of sheep erythrocytes in guinea pig serum deficient in the fourth component of complement. I. Antibody and serum requirements. *J. Immunol.* **111:**1661.
39. May, J. E., and M. M. Frank. 1973. Hemolysis of sheep erythrocytes in guinea pig serum deficient in the fourth component of complement. II. Evidence for involvement of C1 and components of the alternate complement pathway. *J. Immunol.* **111:**1668.
40. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein β1H. *Proc. Natl. Acad. Sci. U. S. A.* **73:**3268.
41. Whaley, K., and S. Ruddy. 1976. Modulation of the alternative complement pathway by β1H globulin. *J. Exp. Med.* **144:**1147.
42. Alper, C. A., F. S. Rosen, and P. J. Lachmann. 1972. Inactivator of the third component of complement as an inhibitor in the properdin pathway. *Proc. Natl. Acad. Sci. U. S. A.* **69:** 2910.
43. Czop, J. K., D. T. Fearon, and K. F. Austen. 1978. Opsonin-independent phagocytosis of activators of the alternative complement pathway by human monocytes. *J. Immunol.* **120:** 1132.
44. Czop, J. K., D. T. Fearon, and K. F. Austen. 1978. Membrane sialic acid on target particles modulates their phagocytosis by a trypsin-sensitive mechanism on human monocytes. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3831.
45. Gigli, I., and R. A. Nelson, Jr. 1968. Complement dependent immune phagocytosis. I. Requirements for C'1, C'4, C'2, C'3. *Exp. Cell Res.* **51:**45.
46. Mayer, M. M. 1978. Complement, past and present. Harvey Lect. **72:**139.
47. Jennings, H. J., A. K. Bhattacharjee, D. R. Bundle, C. P. Kenny, A. Martin, and I. C. P. Smith. 1977. Structures of the capsular polysaccharides of *Neisseria meningitidis* as determined by ^{13}C-nuclear magnetic resonance spectroscopy. *J. Infect. Dis.* **136**(Suppl.)**:**78.
48. Robbins, J. B., G. H. McCracken, E. C. Gotschlich, F. Ørskov, I. Ørskov, and L. A. Hanson. 1974. *Escherichia coli* K1 capsular polysaccharide associated with neonatal meningitis. *N. Engl. J. Med.* **290:**1216.