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Immunology

Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity

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ABSTRACT **Group B streptococci (GBS) cause sepsis and meningitis in neonates and serious infections in adults with underlying chronic illnesses. Specific antibodies have been shown to be an important factor in protective immunity for neonates, but the role of serum complement is less well defined. To elucidate the function of the complement system in immunity to this pathogen, we have used the approach of gene targeting in embryonic stem cells to generate mice totally deficient in complement component C3. Comparison of C3-deficient mice with mice deficient in complement component C4 demonstrated that the 50% lethal dose for GBS infection was reduced by ≈50-fold and 25-fold, respectively, compared to control mice. GBS were effectively killed *in vitro* by human blood leukocytes in the presence of specific antibody and C4-deficient serum but not C3-deficient serum. The defective opsonization by C3-deficient serum *in vitro* was corroborated by *in vivo* studies in which passive immunization of pregnant dams with specific antibodies conferred protection from GBS challenge to normal and C4-deficient pups but not C3-deficient pups. These results indicate that the alternative pathway is sufficient to mediate effective opsonophagocytosis and protective immunity to GBS in the presence of specific antibody. In contrast, the increased susceptibility to infection of non-immune mice deficient in either C3 or C4 implies that the classical pathway plays an essential role in host defense against GBS infection in the absence of specific immunity.**

Group B streptococci (GBS) are the leading cause of bacterial sepsis and meningitis among newborn infants in the United States (1). These organisms also are responsible for up to 50,000 cases per year of infection in pregnant and peripartum women and have been recognized increasingly as a cause of serious infection among nonpregnant adults with diabetes mellitus, cancer, and other chronic illnesses (1–3). Susceptibility to GBS infection among neonates has been correlated with low levels of maternal and cord antibodies to the GBS capsular polysaccharide (4, 5). Human serum samples containing capsular polysaccharide-specific antibodies at >2–3 μg/ml have been shown to opsonize GBS for killing by human neutrophils and to protect mice against experimental GBS infection (6, 7). Antibodies elicited in animals by vaccination with purified capsular polysaccharide coupled to a carrier protein also have been shown to protect mice against lethal GBS challenge (8, 9).

While specific antibody has been demonstrated to play an important part in protective immunity to GBS, the role of the complement system is less clearly defined. Shigeoka *et al.* (10) found that an IgM monoclonal antibody to the type III capsular polysaccharide protected normal neonatal rats from lethal challenge with type III GBS but failed to protect neonatal rats treated previously with cobra venom factor. The investigators concluded that complement was required for IgM antibody-mediated protection. In studies of opsonophagocytosis *in vitro*, Edwards *et al.* (11) found that heat-inactivation of complement in human serum containing capsular polysaccharide-specific IgG antibodies abrogated the ability of the serum to opsonize type III GBS for killing by human neutrophils. A similar finding was reported by Johnston *et al.* (12) as they identified complement component C3 as the important ligand in opsonization of pneumococcus in an *in vitro* assay. These studies suggested that efficient opsonization of GBS by specific antibodies required the participation of serum complement.

Other observations have suggested complement also may be important in host defense against GBS in the absence of capsular polysaccharide-specific antibodies. The incidence of GBS infection among infants is strikingly dependent on age: the incidence is increased among premature infants and infection is rare after 3 months of age (1, 13), despite the fact that most older children and adults lack measurable levels of GBS antibodies (5, 14, 15). One hypothesis proposed to explain the increased susceptibility of young infants is immaturity of the complement system—relatively low levels of one or more complement proteins or decreased expression on phagocytes of complement receptors (16–18). Experimental evidence has suggested a role for the classical pathway in antibody-independent opsonization of GBS. Baker *et al.* (19) found that type Ia GBS could be opsonized by normal adult serum with low or undetectable levels of specific antibody. Further studies suggested that opsonization proceeded via direct activation of the classical pathway (19, 20).

These studies have provided important evidence that serum complement plays a critical part in host defense against GBS, both in the presence and absence of specific antibody. No study, however, has described a definitive model for determining the role of the complement system in immunity to GBS *in vivo* or for the relative importance of the classical and alternative complement pathways in antibody-dependent and antibody-independent immunity. To study these questions directly, we have constructed mice deficient in complement C3 and compared them with mice deficient in complement component C4 to better define the function of complement system in immunity to GBS. Assays of opsonization *in vitro* and of infection *in vivo* provide definitive evidence that serum complement is critical to host defense against GBS both in the presence and absence of specific antibody and indicate a role for both the classical and alternative pathways in immunity to GBS infection. To our knowledge, these are the first published studies utilizing mice genetically deficient in C3 or C4 to examine the function of the complement system in host defense against infection.

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Abbreviations: GBS, group B streptococci or streptococcal; ES cell, embryonic stem cell; Neo, neomycin; CFU, colony-forming unit(s).

MATERIALS AND METHODS

Derivation of C3- and C4-Deficient Mice. Mice deficient in C3 and C4 were constructed by using the approach of homologous recombination in embryonic stem (ES) cells. For the C3 disruption, the targeting vector was assembled from ≈7 kb of genomic DNA isolated from a strain 129 library (isogenic for ES cell line) and inserted into the pPGK/Neo vector. The vector includes a neomycin (Neo) cassette with the PGK promoter and, therefore, allows for selection with G418. To ensure disruption of the C3 coding sequence, ≈600 nt of the gene were deleted and the Neo gene was inserted within an exon. Based on the published cDNA sequence (21), the deleted segment included 364 nt of coding sequence (nt 1850–2214, based on pro-C3 numbering) representing the C-terminal region of the β chain and the N-terminal region of the α chain (aa residues 620–741). This region includes the stretch of basic residues RRRR that is the site for processing the pro-C3 molecule into the two-chain structure found in serum. For transfection, 2×10^7 ES cells [cell line J-1 (22) from Arlene Sharpe, Brigham and Women's Hospital, Boston] were transfected with 20 μg of linear targeting vector pPGK/C3 in an electroporation cuvette (Bio-Rad Pulsar) and treated at 260 V and 500 μF. Colonies surviving selection in G418 (0.4 mg/ml) were picked into 96-well plates, expanded, and analyzed by the technique of Southern (23). Individual clones bearing the mutant C3 allele and that had a normal 40-chromosome karyotype were microinjected into 3.5-day blastocysts (C57BL/6); the embryos were implanted into the uterus of a pseudopregnant female (24). Male chimeric mice were bred with C57BL/6 females to generate offspring heterozygous for the disrupted allele. Agouti offspring were screened by Southern blot analysis as described above to identify inheritance of the mutant C3 allele. To generate homozygous deficient mice, F_1 brother × sister matings were performed. Construction of the C4-deficient mice was performed in a similar fashion and will be described elsewhere (M. Fischer, M.M., S. Han, X. Zhou, J. Xia, O. Finco, G. Kelsoe, and M.C.C., unpublished results).

Bacterial Strains. Two strains of type III GBS were used, COH1 (provided by Craig Rubens, University of Washington, Seattle) and M781 (provided by Carol Baker, Baylor College of Medicine, Houston). Both were originally isolated from infants with invasive GBS infection. Strain M781 produces a larger amount of capsular polysaccharide than strain COH1 and is more highly resistant to complement-mediated opsonophagocytosis *in vitro* (25). GBS were grown from frozen stocks on 5% sheep blood agar or in Todd Hewitt broth.

Lethality Studies in Adult Mice. Adult mice (4–12 weeks of age) were challenged with a single i.p. injection of GBS strain COH1. Groups of 5–12 mice received challenge doses ranging from 10^2 to 10^6 colony-forming units (CFU) for C3- and C4-deficient animals and from 10^3 to 10^7 CFU for control mice. Control mice were C3 heterozygotes from the same breeding stock as the C3-deficient mice. Survival was assessed 3 days after challenge and LD_{50} (dose lethal to 50% of animals challenged) was calculated by the method of Reed and Muench (26) based on data from 29 control animals, 44 C3-deficient animals, and 48 C4-deficient animals.

Opsonophagocytic Assay. Serum from freshly clotted mouse blood was stored at −80°C until use. The opsonophagocytic assay was a slight modification of the procedure described by Baltimore *et al.* (27). Samples contained 1.5×10^6 CFU of GBS grown in broth culture to mid-exponential phase, 3×10^6 human peripheral blood leukocytes, 10% (vol/vol) mouse serum, and, in some experiments, 1% immune rabbit serum as a source of specific antibodies, in a total volume of 500 μl in Eagle's minimum essential medium. Assay mixtures were incubated at 37°C with end-over-end rotation for 60 min. Aliquots were removed at time zero and at 60 min, diluted in sterile water, and spread on plates for quantitative culture. Results were expressed as logarithmic decrease in CFU (i.e., $\log_{10}$ CFU at time zero minus $\log_{10}$ CFU at time 60 min). Control (normal mouse serum) for these experiments was from C57BL/6 mice or from C4 heterozygotes from the same breeding stock as the C4-deficient mice.

Mouse Maternal Immunization/Neonatal Challenge. A maternal immunization/neonatal challenge model (28) was used to test whether specific antibodies could protect newborn mice from GBS challenge. A single 0.5-ml dose of immune rabbit serum [elicited by vaccination with a GBS type III polysaccharide-tetanus toxoid conjugate vaccine (8)] was administered i.p. to pregnant mice 2–12 days prior to delivery. Two days after delivery, the pups were challenged with a single i.p. dose of 5×10^4 CFU of GBS strain M781. Survival was assessed 2 days after challenge. Control mice for these experiments were C4 heterozygotes from the same breeding stock as the C4-deficient mice.

Challenge of Immunized Adult Mice. Groups of adult mice were given two 2-μg doses i.p., 3 weeks apart, of GBS type III polysaccharide-tetanus toxoid conjugate vaccine in 1% alum (8). Antibody response to the type III polysaccharide was measured by ELISA in plates coated with type III polysaccharide conjugated to human serum albumin. Antibody titer was defined as the greatest serum dilution giving an absorbance value >0.3. Six weeks after the first vaccination, mice were challenged with an i.p. injection of 10^6 CFU of type III GBS strain COH1. Six days later, survivors were challenged with an i.p. injection of 10^8 CFU of the same strain. Survival was assessed for 10 days after the initial challenge. Control mice for these experiments were C4 heterozygotes from the same breeding stock as the C4-deficient mice.

RESULTS

Construction of C3-Deficient Mice. The murine C3 locus was disrupted by using the approach of homologous recombination in ES cells (24) (Fig. 1*A*). ES cells (strain J-1) (22) were transfected with the targeting vector pPGK/C3, and colonies bearing the disrupted allele were picked and identified by Southern blot analysis. Two clones, pPGK/C3-A2 and pPGK/C3-B1, were microinjected into embryos and both gave germ-line transmission of the targeted C3 allele. Brother × sister matings of heterozygous offspring yielded the expected ratio of mice homozygous for the targeted C3 locus (Fig. 1*B*). Thus, two distinct lines of mice (i.e., C3A2 and C3B1) homozygous for the mutant C3 allele were developed. Both lines of mutant mice were normal in appearance and were fertile on further breeding. Both lines had undetectable levels of C3 protein in their serum by ELISA and both had no functional activity in a C3 hemolytic assay (data not shown).

Similar methods were used in the development of C4-deficient mice that had normal C3 levels but lacked detectable C4. Development of the C4-deficient mice will be described elsewhere (M. Fischer, *et al.*, unpublished results).

Deficiency of Either C3 or C4 Results in Increased Susceptibility to Lethal GBS Infection. We used a mouse model of lethal systemic infection to test the effect of C3 or C4 deficiency on susceptibility to GBS infection. Because C3 is the primary effector molecule for opsonization, whether through the classical or alternative pathway, we anticipated that C3 deficiency would result in enhanced susceptibility to GBS infection. Previous evidence has indicated that sialic acid residues of the GBS type III polysaccharide inhibit alternative pathway activation (25, 29). Therefore, opsonization in the absence of specific antibody is thought to proceed via the classical pathway. According to this formulation, inability to form the classical pathway C3 convertase because of C4 deficiency would be expected also to impair opsonophagocytosis of GBS, thereby enhancing susceptibility to infection.

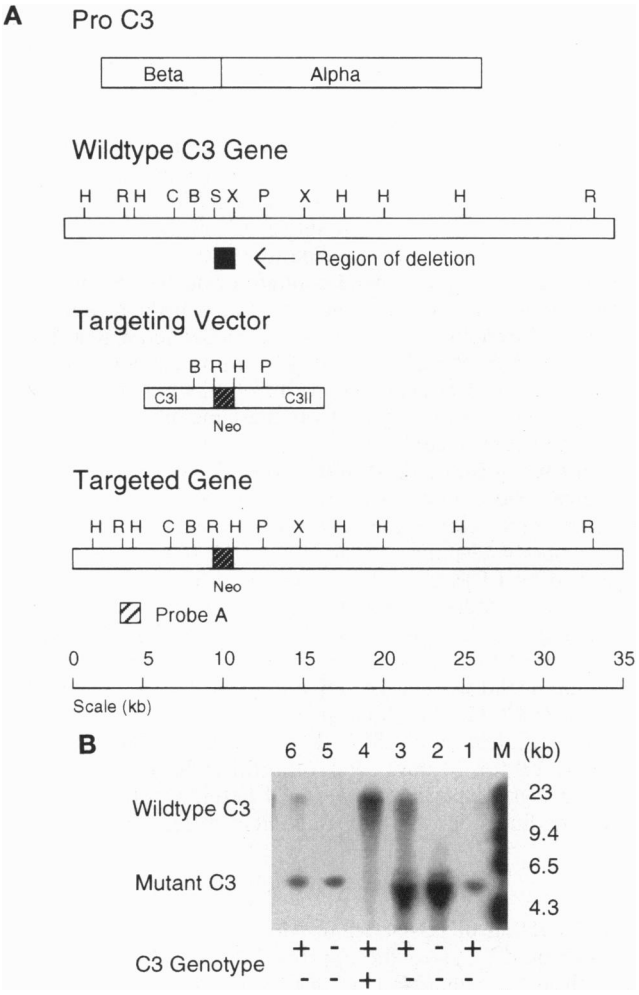

FIG. 1. Targeted disruption of the murine C3 gene. (*A*) Restriction maps of wild-type C3 gene, targeting vector (pPGK/C3), and predicted targeted allele. The C3 gene fragment cloned in the targeting vector contains a deletion spanning amino acids 620–741 (Pro-C3 numbering); the Neo gene was inserted at the site of the deletion. X, *Xba* I; B, *Bst* I; C, *Cla* I; H, *Hin*dIII; P, *Sph* I; S, *Stu* I; R, *Eco*RI. (*B*) Southern blot analysis of *Eco*RI-digested genomic tail DNA from offspring of F_1 mice. Mating F_1 heterozygotes yielded offspring homozygous for the mutant C3 allele as shown by hybridization of probe A to a 6-kb *Eco*RI fragment and absence of hybridization to the wild-type 32-kb fragment.

Adult mice were challenged with a single i.p. dose of type III GBS. In normal mice, sufficient doses of GBS administered in this way result in bacteremia, generally within 48 h of challenge. Bacteremic mice usually die within 3 days of challenge or clear the bacteremia and survive without sequelae. In the current studies, the LD_{50} for control immunocompetent mice was 6.3×10^4 CFU. The LD_{50} was reduced both in C3-deficient mice and in C4-deficient mice to 1.3×10^3 and 2.4×10^3, respectively. All deaths occurred within 3 days after challenge and mice that survived appeared well during 10 days of further observation.

Pathologic Changes Induced by GBS Challenge Were Similar in C3-Deficient, C4-Deficient, and Normal Mice. The enhanced susceptibility of C3- and C4-deficient mice to GBS infection was reflected by a reduced LD_{50}. However, the increased mortality among the complement-deficient mice was limited to the first 3 days after challenge. To investigate whether C3 or C4 deficiency was associated, as well, with more chronic pathology reflecting delayed clearance of the organisms from deep tissues, mice surviving systemic challenge with GBS doses of 10^2 to 10^5 CFU were euthanized 15 days after challenge. Necropsy revealed mild focal hepatitis in two of eight C3-deficient mice and in 1 of 11 C4-deficient mice. These animals had aggregates of mononuclear and/or polymorphonuclear leukocytes within the hepatic sinusoids, sometimes accompanied by degeneration of adjacent hepatocytes. Similar pathologic changes in the liver and inflammatory lesions involving kidney, myocardium, and soft tissues were observed in some normal animals, primarily those that received higher challenge doses. The prevalence and severity of inflammatory lesions was no greater among C3-deficient or C4-deficient mice than among control animals, although chronic pathology could not be assessed in C3- or C4-deficient animals challenged with doses higher than 10^3 or 10^4 CFU, respectively, as these mice did not survive long enough after challenge. The histopathologic findings suggest that although deficiency of one of the complement proteins was an important determinant of mortality during acute infection, it was not a significant contributing factor in chronic inflammatory lesions.

C3 Is Required for Antibody-Mediated Opsonophagocytic Killing. Since GBS, like other Gram-positive bacteria, are resistant to direct lysis in serum, their clearance from the blood depends largely on ingestion and killing by phagocytic leukocytes. We utilized an *in vitro* assay to test the ability of serum from C3- or C4-deficient mice to support opsonophagocytic killing by human blood leukocytes. By using as a target strain COH1, a type III GBS strain with a moderate amount of capsular polysaccharide, normal mouse serum mediated a 1.0 $\log_{10}$ reduction in CFU in the presence of 1% immune rabbit serum added as a source of specific antibody (Fig. 2). Antibodies in the immune serum were elicited by vaccination of rabbits with a type III capsular polysaccharide-tetanus toxoid vaccine and are primarily of the IgG isotype (8). Opsonization with serum from C3-deficient mice and 1% immune rabbit serum resulted in only a 0.2 $\log_{10}$ reduction in CFU. In contrast, opsonization with C4-deficient serum and specific antibody gave similar results to those observed with normal mouse serum, a 1.2 $\log_{10}$ decline in GBS CFU. Similar results were obtained both for C3- and C4-deficient sera using the highly encapsulated type III strain M781 as a target, rather than strain COH1 (Fig. 2). These results indicate that C3 is essential for efficient opsonophagocytic killing of GBS, even in the presence of specific antibody. That opsonophagocytic killing proceeded normally in C4-deficient serum implies that the classical pathway is not required but rather that antibody-mediated opsonization can proceed via the alternative pathway.

In the absence of specific antibody, none of the mouse sera tested supported killing of the highly encapsulated strain

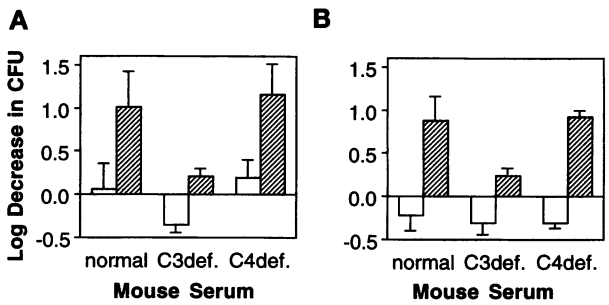

FIG. 2. Opsonophagocytic killing of type III GBS opsonized in serum from C3-deficient (C3def.), C4-deficient (C4def.), or normal mice. Moderately encapsulated (strain COH1) (*A*) or highly encapsulated (strain M781) (*B*) type III GBS were incubated with 10% serum from normal, C3-deficient, or C4-deficient mice as a complement source and with (hatched bars) or without (open bars) 1% immune rabbit serum as a source of specific antibodies. The results are expressed as $\log_{10}$ decrement in viable bacteria after 1 h of incubation and represent mean ± SEM of duplicate determinations in three experiments.

M781. With the moderately encapsulated strain COH1 as a target, a slight reduction in CFU was observed in the presence of normal or C4-deficient serum (0.1–0.2 $\log_{10}$), while 0.4 $\log_{10}$ increase in CFU was observed in the presence of C3-deficient serum.

Antibody-Mediated Protection Against GBS Infection in Neonatal Mice Requires C3 but not C4. Results of the opsonophagocytic assays indicated that C3 and specific antibody were required for efficient opsonization of type III GBS *in vitro*. To test the importance of C3 in antibody-mediated bacterial clearance *in vivo*, we utilized a maternal immunization/neonatal challenge model of GBS infection. Pregnant dams were passively immunized with immune rabbit serum. Previous studies using this model have demonstrated that IgG antibodies are transported across the placenta and confer protective immunity to the newborn pups (9, 28, 30). As expected, control pups of passively immunized dams were protected against lethal challenge on day 2 of life: 14 of 15 pups survived and none had GBS bacteremia detected 2 days after challenge (Fig. 3). Similarly, C4-deficient pups also were protected: 15 of 16 survived challenge and all 15 survivors had negative blood cultures 2 days after challenge. This result implies that the classical pathway is not required for antibody-mediated immunity in this model. In contrast, 15 of 20 C3-deficient pups died within 48 h of challenge ($P < 0.0001$ for comparison to control group, Fisher's Exact Test) and 4 of the 5 survivors had blood cultures positive for GBS 2 days after challenge. Thus, impaired antibody-mediated clearance of GBS by the C3-deficient mice was manifested by decreased survival after challenge and persistent bacteremia. These results are consistent with those of the *in vitro* opsonophagocytic assays. They imply that C3 is critical for antibody-mediated clearance of GBS and that antibody-mediated opsonization can proceed efficiently via the alternative pathway.

To test the importance of the complement system in antibody-mediated immunity in adult mice, groups of mice were immunized with GBS type III polysaccharide-tetanus toxoid conjugate vaccine. Titers of antibodies to the type III polysaccharide measured 4 weeks after the primary immunization were similar in the three groups, 1:200 in the C3-deficient and C4-deficient mice and 1:400 in the control mice. Six weeks after the primary immunization, the mice were challenged with 10^6 CFU of type III GBS, a dose ≈500 times the LD_{50} for nonimmune C3-deficient or C4-deficient mice. Eight of 10 C3-deficient mice and 8 of 9 C4-deficient mice survived challenge, compared to 10 of 10 control mice. When the same mice were challenged with 10^8 CFU, 2 C3-deficient mice survived 2 days after challenge compared to 4 C4-deficient and 4 control mice. At 3 days, 1 C3-deficient mouse survived compared to 2 C4-deficient and 2 control mice. These results indicate that, in contrast to the findings in neonatal mice, antibody conferred some degree of protective immunity to C3-deficient adult mice. As with the neonatal mouse model, however, the survival curves after high-dose challenge suggested the C3-deficient adults were somewhat more susceptible than the C4-deficient or normal mice, although the number of animals was too small to determine whether this difference was significant.

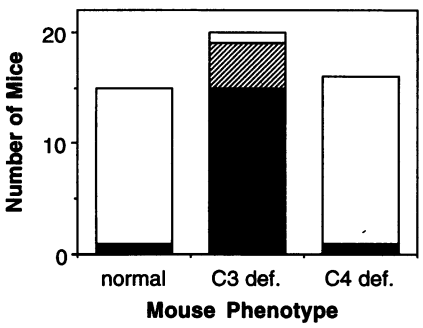

FIG. 3. Maternal immunization/neonatal challenge model of GBS infection. Pregnant dams were passively immunized with immune rabbit serum. After delivery, the pups were challenged with a lethal dose of type III GBS. Two days after challenge, mortality was determined and survivors were assessed for bacteremia by blood culture. Deaths are indicated by solid bars, bacteremic survivors are indicated by hatched bars, and healthy (nonbacteremic) survivors are indicated by open bars.

DISCUSSION

These studies in C3- and C4-deficient mice provide direct evidence that the complement system plays a central role in immunity to GBS. The enhanced susceptibility of C4-deficient and C3-deficient mice to GBS infection implicates the classical pathway in "innate" immunity, i.e., resistance to infection in the absence of specific antibody. Complement activation by bacteria generally has been thought to proceed via the alternative pathway. However, highly encapsulated strains of type III GBS have been shown previously not to activate the alternative pathway in the absence of specific antibody (29). Failure to activate the alternative pathway appears to reflect the inhibitory effect of sialic acid, present as terminal side chain residues of the type III capsular polysaccharide. Particles with sialic acid-rich surfaces are thought to increase the affinity of factor H for surface-bound C3b, resulting in cleavage of C3b by factor I and interruption of the C3 amplification loop (31, 32). The relevance of this mechanism to GBS has been supported by the finding that removal of sialic acid from the type III capsule by neuraminidase treatment or by genetic manipulation produces organisms that activate the alternative pathway (25, 29, 33). Such sialic acid-deficient organisms can be opsonized via the alternative pathway by human serum lacking specific antibody, while organisms bearing the native (sialylated) capsular polysaccharide cannot. The finding in the present investigation that C4-deficiency increased susceptibility to GBS infection to a similar extent as C3-deficiency supports the hypothesis that the classical, and not alternative, pathway is primarily involved in antibody-independent humoral immunity to this organism.

Experiments examining antibody-dependent immunity to GBS also demonstrated a role for the complement system. For many organisms including encapsulated bacteria like GBS, complement appears to play a synergistic, and in some cases, essential role in antibody-mediated opsonophagocytosis. C4-deficient guinea pigs and cobra venom factor-treated guinea pigs both had marked impairment in bloodstream clearance of pneumococci preopsonized with capsule-specific IgG antibodies, consistent with a requirement for the classical pathway for efficient antibody-mediated opsonophagocytosis (34). For other encapsulated bacteria, however, the classical pathway does not appear to be required for deposition of opsonic C3b in the presence of specific antibody. Evidence has been presented to support alternative pathway activation in opsonization by specific antibodies of *Staphylococcus aureus* (35), *Bacteroides fragilis* (36), and GBS (11). For type III GBS, inhibition of alternative pathway activation can be overcome by specific antibodies, resulting in deposition of C3b on the bacterial surface and opsonophagocytosis by neutrophils (11, 25, 29). In the present investigation, we found that capsular polysaccharide-specific antibodies protected C4-deficient mice, but not C3-deficient mice, in a maternal immunization/neonatal challenge model. In contrast, challenge of immunized adult mice indicated that specific antibodies conferred at least partial protection to C3-deficient adult mice, C4-deficient mice, and normal controls. These *in vivo* results were corroborated by opsonophagocytic assays in which C4-deficient, but not C3-deficient, serum supported antibody-dependent opsonophagocytic killing of GBS. Thus, these data support the

hypothesis that antibody-mediated clearance may proceed via the alternative pathway.

Based on these results, we conclude that the classical pathway participates in early bloodstream clearance of GBS in the absence of specific antibodies. In the neonatal mouse model, antibody-mediated immunity also requires C3, but not C4, supporting involvement of the alternative pathway in antibody-dependent opsonophagocytosis. The generality of findings in the mouse model may be limited by experimental parameters such as the route of challenge, the relative resistance of mice to GBS infection, and differences between mouse and human complement systems. These limitations notwithstanding, the present study provides further direct evidence that complement plays a central role in host defense against GBS. Mice with genetic deficiencies in specific complement proteins will provide useful model systems for further study of the mechanisms of host defense against this and other microbial pathogens.

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