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# Protection of Neonatal Mice from Group B Streptococcal Infection by Maternal Immunization with Beta C Protein

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**Group B streptococci (GBS) cause the majority of cases of neonatal sepsis and meningitis in the United States. Immunization of women of childbearing age is one strategy under consideration for the prevention of neonatal disease. The beta C protein, a 130-kDa antigen present in many clinical isolates of GBS, was purified from GBS by extraction into sodium dodecyl sulfate (SDS)-containing buffer, preparative SDS-polyacrylamide gel electrophoresis, and electroelution. Purified beta C protein antigen (25 μg) with Freund's adjuvant was used to immunize rabbits. Rabbits developed enzyme-linked immunosorbent assay titers of >1:1.6 × $10^6$, and sera from immunized rabbits were administered to pregnant mice. Their neonatal pups were then challenged with a strain of GBS expressing beta C protein; 68% of these pups were protected by immune antiserum, whereas no controls were protected ($P < 0.001$). The immune serum (diluted 1:100) facilitated opsonophagocytic killing of GBS strains expressing the beta C protein but not those that do not express the antigen (mean log kill ± standard deviation = 0.71 ± 0.8 $\log_{10}$ CFU for beta$^+$ strains and 0.09 ± 0.2 for beta$^-$ strains; $P = 0.02$). In subsequent experiments, adult female mice were actively immunized with two doses of 2, 5, or 10 μg of beta C protein 2 months prior to mating. One- to two-day-old offspring of these dams were challenged with GBS and were protected in a dose-dependent manner, with 96% survival in the high-dose (10-μg) group and 20% survival in a sham-immunized control group ($P < 0.001$). Thus, active immunization of mice with the GBS beta C protein confers protection against lethal infection with beta$^+$ GBS to their offspring.**

*Streptococcus agalactiae* (group B streptococci [GBS]) is the most common cause of serious bacterial infection during the neonatal period (1). This organism is a leading contributor to infant mortality, with infection occurring at rates of 2 to 5/1,000 live births in the United States (1). Despite aggressive and improving medical therapy, mortality and serious morbidity remain unacceptably high for these infections, and prevention remains an important goal. Immunization of women of childbearing age has been proposed as a strategy for the prevention of neonatal sepsis on the basis of the finding that antibodies to the type-specific GBS capsular polysaccharides passed transplacentally prevent serious neonatal infection (2, 3). The most extensively studied antigens for potential GBS vaccine use have been the capsular polysaccharides. However, the rates of immunogenicity of the polysaccharide antigens are unacceptably low unless the antigens are conjugated to a protein carrier (4). In addition, in order to be clinically useful, a polysaccharide vaccine for GBS would need to include each of the four major type-specific polysaccharides.

Another set of antigens were shown by Lancefield and coworkers to elicit protective antibody: the C proteins (18). Initially recognized in Ib and Ic serotypes of GBS and called the Ibc protein (31), these protein antigens have been found to be expressed independently from capsular antigens (17). The C proteins are found in most strains of GBS except for those of serotype III. Two components of the C protein complex were recognized initially on the basis of different protease sensitivities (31). The alpha, or trypsin-resistant, antigen is expressed as a series of proteins that exhibit a laddering motif on Western blots (immunoblots) and exhibit diversity in molecular weight between strains (19). Using antibodies induced by immunization with the cloned gene product, we have demonstrated that monoclonal antibody to this antigen is protective (20, 21). The beta, or trypsin-sensitive, C protein is expressed as a single 130-kDa protein that binds to human immunoglobulin A (IgA) (although smaller, non-IgA-binding variants in some strains have been described) (9, 27, 28). We have also demonstrated mouse protection by using antiserum to the cloned beta C protein (21); others have demonstrated that this antigen will elicit mouse-protective antibody in rabbits (7). Gamma and delta C proteins have also been described but are less fully characterized (10, 12). C proteins have been reported to have a role in virulence, possibly by inhibiting opsonophagocytic killing via reduction of complement deposition (19, 23–25).

In order to examine the potential of C proteins as vaccines for the prevention of neonatal GBS sepsis, we report here the purification of the beta C protein and examination of its immunogenicity and ability to elicit opsonic and mouse-protective antibodies. We examine the ability of maternal immunization with the beta C protein to induce antibodies which, via transplacental antibody passage, protect neonatal mice from lethal challenge with GBS.

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## MATERIALS AND METHODS

**Bacterial strains.** GBS were obtained either from the collection of the Channing Laboratory or from hospitals associated with the Baylor College of Medicine and have been previously described (19). Capsular type Ia included

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strains 090 and S5 (alpha$^-$, beta$^-$), S18 and S19 (alpha$^-$, beta$^+$), S27 (alpha$^+$, beta$^-$), and A909 and S9 (alpha$^+$, beta$^+$). Capsular type Ib included strains H36B, S13, S37, S38, S39 and S41 (alpha$^+$, beta$^+$), and S7, S14, S42, and S43 (alpha$^-$, beta$^+$). Capsular type II included strains 18RS21 (alpha$^-$, beta$^-$), S20 (alpha$^+$, beta$^-$), and S54 and S56 (alpha$^+$, beta$^+$). Capsular type III included strains M732, S23, and S24 (alpha$^-$, beta$^-$).

**Purification of beta C protein by preparative SDS-PAGE and electroelution.** The detergent-extracted beta C protein of GBS migrates as a single 130-kDa band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels; this property was exploited for purification of the antigen. Strain S7, which was previously characterized as Ib/C (alpha$^-$, beta$^+$) (19), was grown overnight in 500 ml of Todd-Hewitt broth ($A_{650} \approx 0.6$) and washed in phosphate-buffered saline; the pellet was resuspended in an equal volume (≈1 ml) of SDS sample buffer (125 mM Tris [pH 6.8], 10% SDS, 20% glycerol, 0.04% bromophenol blue). This suspension was extracted in a boiling water bath for 5 min and pelleted in a microcentrifuge for 2 min; 0.25 ml of supernatant was applied to each of four SDS–10% polyacrylamide minigels (1.5 by 60 by 80 mm), and electrophoresis was performed. A 1-cm strip was cut from each side of each gel and stained with Coomassie brilliant blue. These strips were then realigned with the minigel, and a 0.25-cm horizontal band corresponding to the 130-kDa beta C protein was removed from the unstained portion of each gel. These were subjected to electroelution in an Elutrap apparatus (Schleicher and Schuell) at 100 V overnight. The gel slices were stained after electroelution to confirm removal of the protein. The eluent (~1 ml) was collected and lyophilized. Protein was assayed by the bicinchoninic acid method (Pierce). Purity was assessed by SDS-PAGE and Western blot with monoclonal antibody 3E7 specific for the beta C protein, by immunoblot with human myeloma IgA (100 μg/ml; kindly provided by A. Plaut, Boston, Mass.) as previously described (19), and by Western blot with rabbit antiserum raised by the method of Lancefield et al. (18) to intact formalinized GBS strains H36B (Ib/C, alpha$^+$, beta$^+$), 090 (Ia, C protein negative), and M732 (III, C protein negative) at a 1:5,000 dilution, and probed with goat anti-rabbit antibodies conjugated to alkaline phosphatase (8).

**Rabbit antiserum.** Three rabbits were immunized by subcutaneous injection; the first dose (25 μg) was emulsified in 0.5 ml of complete Freund's adjuvant, and the subsequent doses (days 21 and 42, 25 μg) were emulsified in 0.5 ml of incomplete Freund's adjuvant. Serum was collected before immunization on each day and again 2 weeks after the last dose. Specificity of the immune antiserum was tested by Western blot analysis of intact GBS strain S7.

**ELISA.** Antibody to the beta C protein was measured by enzyme-linked immunosorbent assay (ELISA) as described previously (19). The titer was determined by serial twofold dilutions (from a starting dilution of 1:100 [rabbits] or 1:400 [mice]) as the greatest dilution with an $A_{405}$ of ≥0.2 after 30 min of development.

**Opsonophagocytic assay.** The functional capacity of antibodies to C proteins of GBS was assessed by an opsonophagocytic assay (5) measuring in vitro killing of GBS. In brief, a 300-μl volume of human polymorphonuclear leukocytes (PMNs) (approximately $3 \times 10^6$ cells) was mixed with the test GBS strain (approximately $1.5 \times 10^6$ CFU), 50 μl of human serum (as a complement source), and 100 μl of antiserum diluted 1:100. Viable GBS cells were enumerated as 10-fold dilutions on blood agar plates immediately and at 60 min, and the difference was calculated as the amount of killing. The assay was repeated in the absence of antibody. The result is reported as the "log kill," which is the difference ($\log_{10}$ CFU) between killing with and without antibody for at least two determinations per strain. Human serum was prepared for use as a complement source by absorption on ice for 30 min with organisms from one blood agar plate of the GBS serotype of the strain assayed. The bacteria were removed by centrifugation and filter sterilization, and the absorbed serum was stored in aliquots at −80°C.

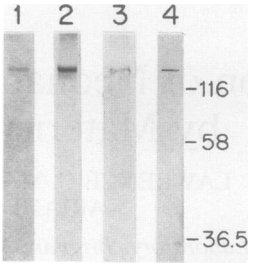


FIG. 1. Analysis of beta C protein of group B streptococci. Lane 1, purified beta C protein visualized by staining with Coomassie brilliant blue; lane 2, Western blot probed with mouse monoclonal antibody 3E7 specific for the beta C protein; lane 3, Western blot probed with antiserum raised to intact GBS strain H36B (Ib/C, alpha$^+$, beta$^+$). Lane 4, Western blot of SDS extract of GBS strain S7 (source of purified beta C protein) probed with rabbit antiserum raised to the purified beta C protein. Molecular mass standards are in kilodaltons.

**Neonatal mouse protection.** A neonatal mouse model of GBS infection has been developed (26) and was used in this study to assess the protective efficacy of passive and active immunization to the beta C protein of GBS in pups challenged with a beta C protein-positive strain of GBS, A909 (Ia/C, alpha$^+$, beta$^+$). For passive-immunization experiments, pregnant CD-1 outbred mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were injected intraperitoneally with 0.1 ml of pooled rabbit serum as described above. Pregnant control mice were injected with rabbit serum either obtained prior to immunization or taken from rabbits immunized with tetanus toxoid (22). For active-immunization experiments, 8-week-old female CD-1 mice were immunized prior to breeding with two intraperitoneal injections 21 days apart of 0, 2, 5, or 10 μg of purified beta C protein. Pups were challenged with $5 \times 10^4$ CFU of GBS type Ia/C strain A909 within 48 h of birth. The challenge dose was administered intraperitoneally with a tuberculin syringe with a 27-gauge needle in a total of 0.05 ml of Todd-Hewitt broth. The challenge dose for each strain was determined to be lethal for 80 to 90% of nonimmune pups of the same age. The number of pups that survived GBS infection was assessed 48 h after challenge.

## RESULTS

**Purification of GBS beta C protein.** The yield of beta C protein from four preparative minigels was ~25 μg. Ten separate extractions were performed, and the antigen was pooled prior to analysis and immunization. SDS-PAGE of the resultant extract revealed >90% purity of a single 130-kDa band (Fig. 1, lane 1). The 130-kDa band reacted in Western blots with monoclonal antibody 3E7, which recognizes the beta C protein (19), and with antiserum raised to

TABLE 1. Opsonophagocytic killing of prototype strain A909 by rabbit hyperimmune antiserum raised to beta C protein[a]

| Dilution | Killing at 60 min (mean $\log_{10}$ CFU ± SD) with serum | |
|---|---|---|
| | Preimmune | Immune |
| 1:10 | 0.39 ± 0.6 | 1.8 ± 0.3 |
| 1:100 | 0.20 ± 0.1 | 1.6 ± 0.2 |
| 1:1,000 | 0.09 ± 0.4 | 1.3 ± 0.01 |

[a] Bacteria were incubated with rabbit serum, human PMNs, and complement (10%) and enumerated at 0 and 60 min. Hyperimmune antiserum raised to the whole bacteria and diluted at 1:10 induced killing of 1.6 ± 0.2 ($\log_{10}$ CFU difference at 0 and 60 min in the presence of antibody − $\log_{10}$ CFU difference at 0 and 60 min in the absence of antibody).

intact type Ib/C strain H36B (alpha$^+$, beta$^+$) (Fig. 1, lanes 2 and 3) but failed to react with antiserum to type Ia strain 090 (alpha$^-$, beta$^-$) or type III strain M732 (alpha$^-$, beta$^-$).

**Analysis of antibodies resulting from rabbit immunization by ELISA and opsonophagocytic assay.** Serum from each rabbit immunized with beta C protein achieved a peak ELISA titer of >1:1.6 × $10^6$, a >4,000-fold increase relative to preimmunization levels. The antiserum reacted specifically with the 130-kDa protein in an extract of the intact S7 GBS strain in SDS sample buffer (Fig. 1, lane 4). The ability of the rabbit antiserum to the beta C protein to opsonize GBS for killing by PMNs was studied with an opsonophagocytic assay. Initially, dilutions of the rabbit antiserum ranging from 1:10 to 1:1,000 were used to study killing of the prototype Ia/C strain A909 (Table 1). Bacterial killing was significantly enhanced, compared with that induced by preimmunization serum, by the hyperimmune serum at dilutions of 1:10 to 1:1,000 and exceeded 95% or 1.2 $\log_{10}$ CFU. The degree of antibody-mediated killing was comparable to that seen with hyperimmune serum raised to the intact bacteria at a 1:10 dilution.

In order to test the biologic activity of beta-specific antiserum against a variety of clinical isolates, opsonophagocytic killing of GBS strains expressing differing C-protein and capsular phenotypes was assessed. Twenty-four GBS isolates (7 type Ia, 10 type Ib, 4 type II, and 3 type III) were employed. Nine strains expressed both beta and alpha C proteins, seven expressed the beta C protein alone, two expressed the alpha C protein alone, and six expressed neither alpha nor beta C protein. Beta$^+$ strains were killed significantly more effectively in the presence of the immune rabbit serum than the beta$^-$ strains were (Fig. 2 and Table 2). These results indicate that most strains expressing the beta C protein were rendered susceptible to opsonization and phagocytic killing in the presence of antibody to the beta antigen, whereas beta$^-$ strains remained resistant to killing. Interestingly, strains that expressed both the alpha and beta C proteins were less susceptible to killing in the presence of beta-specific antibody than strains expressing only the beta C protein (Table 2). Expression of the alpha C protein apparently renders strains relatively resistant to opsonophagocytic killing by anti-beta-C-protein-specific serum. No killing of bacteria occurred when complement was first heat inactivated at 56°C for 30 min.

**Neonatal mouse protection by passive maternal immunization.** In order to assess the protective efficacy of the rabbit antisera, pregnant mouse dams received preimmune or day 70 pooled immune serum from rabbits 1 to 5 days before delivery of pups. The pups were then challenged with 5 × $10^4$ CFU of GBS strain A909 (type Ia/C; of heterologous capsular type from the strain from which the beta C protein was purified). Pup survival was noted at 48 h: 17 of 25 pups (68%) born to mothers immunized with hyperimmune rabbit serum survived, compared with 0 of 16 pups (0%) born to mothers who received saline ($P < 0.001$, Fisher's exact test) and 4 of 28 (14%) born to mothers who received hyperimmune rabbit antiserum to tetanus toxoid ($P < 0.001$).

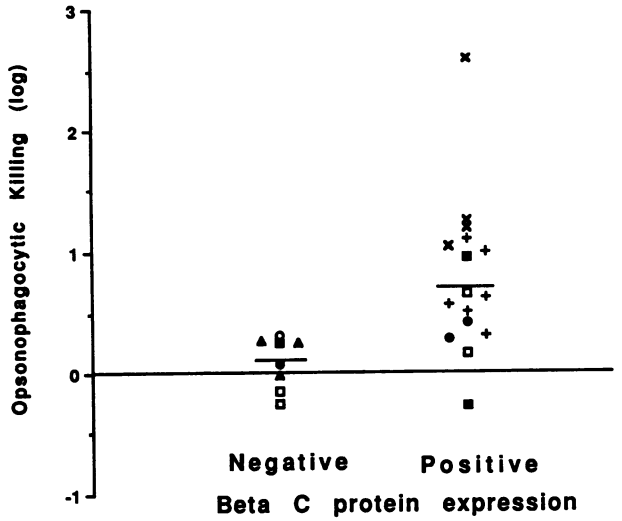


FIG. 2. Augmentation of opsonophagocytic killing ($\log_{10}$ CFU) of group B streptococcal strains by rabbit antiserum raised to beta C protein. Sixteen beta$^+$ strains are compared with 8 beta$^-$ strains (mean of at least two determinations per strain). Symbols for serotypes of GBS strains: □, Ia alpha$^-$; ■, Ia alpha$^+$; ×, Ib alpha$^-$; +, Ib alpha$^+$; ○, II alpha$^-$; ●, II alpha$^+$; ▲, III alpha$^-$. The horizontal bar indicates the mean opsonophagocytic killing for each group.

**Neonatal mouse protection by active maternal immunization.** In order to determine the efficacy of active immunization with beta C protein in adult female mice for prevention of GBS infection in neonates, female CD-1 outbred mice were immunized with 2, 5, or 10 μg of purified beta antigen in 0.5 ml of deionized $H_2O$ intraperitoneally at 0 and 21 days. Antibody response was determined by ELISA before each immunization, 14 days after completion of immunization,

TABLE 2. Opsonophagocytic killing of group B streptococcal strains by rabbit hyperimmune antiserum raised to beta C protein[a]

| C-protein phenotype[b] | No. of strains | Killing at 60 min (mean $\log_{10}$ CFU ± SD)[c] |
|---|---|---|
| Beta$^-$ (all) | 8 | 0.09 ± 0.2[d] |
| Alpha$^+$ | 3 | —[e] |
| Alpha$^-$ | 5 | — |
| Beta$^+$ (all) | 16 | 0.71 ± 0.8[d] |
| Alpha$^+$ | 9 | 0.49 ± 0.6[f] |
| Alpha$^-$ | 7 | 1.16 ± 1.1[f] |

[a] The bacterial strain was incubated in the presence of PMNs, human serum as a source of complement, and rabbit antibeta serum diluted 1:100.
[b] Presence or absence of alpha and beta C protein as determined by Western blot with monoclonal antibody.
[c] Calculated as $\log_{10}$ CFU difference at 0 and 60 min in the presence of antibody − $\log_{10}$ CFU difference at 0 and 60 min in the absence of antibody.
[d] $P = 0.02$ (Student's $t$ test).
[e] Insufficient number of strains in this subgroup to be significant.
[f] $P = 0.01$ (Student's $t$ test).

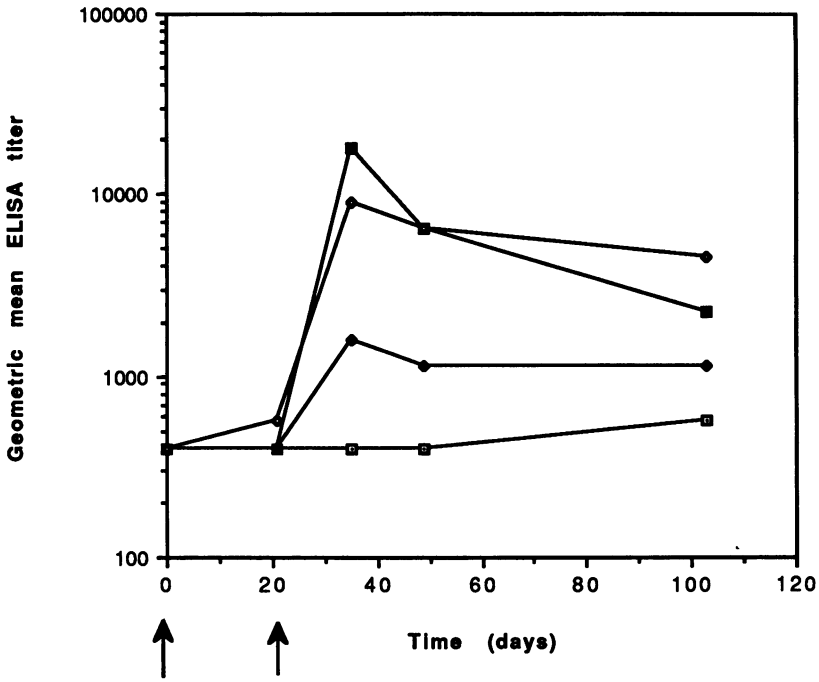


FIG. 3. Maternal antibody response to immunization with beta C protein. Geometric mean ELISA titer of maternal mouse serum versus time. Pups were born between days 98 and 105. ⊡, 0 μg (control); ◆, 2 μg; ◘, 5 μg; ◆, 10 μg; ↑, immunization.

and within 5 days postpartum. Antibody response (Fig. 3) was dose dependent, was maximal at 2 weeks after the second immunization, and then declined gradually. The females were mated and delivered pups between 80 and 100 days after completion of immunization. The pups were challenged as for the passive-immunization experiments. Protection of neonates was also dose dependent (Table 3) and was significantly higher for each treatment group than for the sham-immunized controls. Nearly all pups in the 10-μg group were protected (96% survival).

## DISCUSSION

A variety of approaches for the prevention of neonatal GBS sepsis in humans have been explored. Among these have been antibiotic treatment of mother and/or neonate, immunization of the mother with type-specific polysaccharide, and treatment of neonates with immunoglobulins containing antibodies to GBS polysaccharides (13, 14). A polysaccharide-tetanus toxoid conjugate vaccine against type III GBS elicited protective antibodies in animals (30) and is currently being evaluated as a potential vaccine candidate for human subjects. It is believed that this glycoconjugate vaccine will have enhanced immunogenicity with respect to the polysaccharide antigen. Since protein antigens elicit immunity in a T-cell-dependent manner, these antigens are often better immunogens than polysaccharides and also elicit boostable antibody responses. Since the C proteins are protective antigens, they might be expected to be effective vaccines without further modification.

TABLE 3. Neonatal mouse survival following maternal immunization with beta C protein[a]

| Immunizing dose (μg) | No. of pups (no. of dams) | No. (%) surviving 48 h |
|---|---|---|
| 0 | 45 (3) | 9 (20) |
| 2 | 24 (2) | 12 (50)[b] |
| 5 | 38 (3) | 34 (89)[c] |
| 10 | 24 (2) | 23 (96)[d] |

[a] Groups of five female mice were immunized with beta C protein at the indicated dose and given boosters with the same dose at 21 days of age. Offspring born to these mice during a 1-week period were challenged within 48 h of birth with $5 \times 10^4$ GBS, and survivors were counted 48 h after challenge.
[b] $P = 0.01$ compared with 0-μg control (Fisher's exact test).
[c] $P < 0.001$ compared with 0-μg control (Fisher's exact test).
[d] $P < 0.001$ compared with 0-μg control (Fisher's exact test).

We demonstrate here that antibody to the purified beta C protein is both opsonic and protective against strains of GBS that express the antigen. We have previously shown that *Escherichia coli* clones expressing this antigen are capable of evoking mouse-protective antibodies in rabbits (21). Bevanger and Naess have raised rabbit antisera to acid-extracted antigens of GBS and demonstrated the protective capacity of these sera in adult mice (7). However, the acid-extracted beta antigen they described exhibited several molecular weights and had a ladder appearance similar to that of the alpha C protein (6). Since it was purified by affinity chromatography with polyclonal antisera raised to streptococcal extracts, it may have contained other antigens.

Valtonen et al. described a 14-kDa protein that elicits protective antibody in adult mice (29). This antigen was believed to be a fragment of a larger protein, since antiserum raised to it reacted with several larger proteins in immunoprecipitation. We have recently found that this antiserum to the 14-kDa protein reacts with the cloned beta C protein but not with the cloned alpha C protein in Western blots (unpublished observation) and therefore is most likely a fragment of the beta C protein that contains one or more protective epitopes.

The beta C protein purified by us was detergent extracted at neutral pH and migrates predominantly as a single 130-

kDa protein band on SDS-PAGE gels. Its identity was confirmed by reactions with monoclonal antibody 3E7 and human myeloma IgA. Rabbit antiserum raised to this purified protein product exhibited an extremely high ELISA titer, protected neonatal mice from infection by passive immunization of dams during pregnancy, and opsonized most beta-C-protein-positive but not beta-C-protein-negative strains of GBS for killing by PMNs independent of capsular type. There is variability among beta$^+$ strains in the extent of killing induced by the beta-specific antiserum (Fig. 2), which may be attributable to effects of capsule size, growth rate, or other unmeasured differences between these clinical isolates of GBS. Opsonic activity of the antiserum was apparent against the prototype A909 strain of GBS at dilutions as high as 1:1,000. We have previously demonstrated that strains that express the alpha C protein are more resistant to opsonophagocytic killing in the absence of specific antibody than strains that lack this protein (19). In this study, we showed that expression of the alpha C protein renders GBS less susceptible to opsonization and killing even in the presence of antibodies to beta C protein. These results further support the potential role of the alpha C protein in evading host immunity, possibly by inhibiting the deposition of complement on the bacterial surface (25).

Active immunization in adult female mice elicited a brisk antibody response, as measured by ELISA after a single boost. The antibody response was dose dependent throughout the range (2 to 10 μg) tested and persisted at high levels (ELISA titer, >1:1,000) for >80 days after the second and final boost. Neonates born to these mice were protected against GBS infection, and the level of protection was related directly to the maternal antibody titer, as determined by ELISA, which correlated with the dose of antigen used for immunization.

These findings support the utility of C proteins as vaccines or components of vaccines. While C proteins are present in approximately 60% of all GBS isolates, they tend to occur more frequently in strains which cause early-onset neonatal disease (11). It is perhaps in these cases, in which infection frequently occurs in utero through ruptured or compromised membranes and which currently have the highest mortality rates, that maternal immunization can play the greatest role. One potential use of a C protein would be as a carrier protein in a glycoconjugate vaccine. Since C proteins occur rarely in type III strains, a conjugate vaccine composed of type III polysaccharide and one or more C proteins could elicit immunity to over 90% of disease-causing GBS isolates without necessitating a multivalent vaccine.

One drawback to the usefulness of the beta C protein is that it is expressed less frequently than the alpha C protein in clinical isolates (~40% of C-protein-positive isolates versus 90% for alpha C protein) (17, 19). Thus, ideally, it might be preferable to construct a vaccine by using the alpha C protein as well as the beta C protein to cover as many isolates as possible. Another theoretical drawback to use of the beta C protein as a human vaccine is its ability to bind human IgA via the Fc portion of the immunoglobulin. This binding ability could conceivably modulate immunity in a fashion which is difficult to predict. Since variant strains of GBS which express immunoreactive but non-IgA-binding beta C proteins exist, it may be possible to exploit one of these strains to develop a vaccine which contains the antigenic and/or immunogenic domains of the beta C protein but not the IgA-binding characteristic (9). Two groups have recently mapped and sequenced the gene encoding the beta C protein of GBS, and both have localized IgA binding to specific loci within the gene (15, 16). It may therefore be possible to genetically manipulate this protein to separate IgA binding and antigenic domains. It would then be feasible to use only the antigenic region of the beta C protein as a vaccine component.

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