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A Monoclonal Antibody Identifies a Protective C-Protein Alpha-Antigen Epitope in Group B Streptococci

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Group B streptococci (GBS) are the leading causes of neonatal sepsis and meningitis in the United States, with a high rate of fatality and serious morbidity despite appropriate therapy. The C-protein antigens of GBS appear to be important in immunity to experimental infection, yet these antigens remain incompletely characterized with respect to their number, structure, and function. None of these proteins has yet been purified to homogeneity. We have developed a novel method for extraction of surface proteins from the A909 (Ia/c) strain of GBS by using mutanolysin. Antibodies raised in rabbits against these partially purified proteins conferred passive protection to lethal GBS infection in mice challenged with a GBS strain expressing C proteins with a heterologous capsule type. In addition, mouse monoclonal antibodies were produced and identified by reactivity with the mutanolysin-extracted proteins. One of these monoclonal antibodies (4G8) identifies an epitope on the alpha-antigen of the GBS C proteins (identified by protease susceptibility and mouse protection). On sodium dodecyl sulfate-polyacrylamide gels, this epitope appears as a series of regularly spaced bands ranging in apparent molecular mass from 160,000 to 30,000 Da. The monoclonal antibody 4G8 induces opsonic killing of GBS and protects mice from lethal challenge with GBS. Thus, the 4G8 monoclonal antibody identifies a fully protective epitope on the C-protein alpha-antigen of GBS.

Group B streptococci (GBS) are the leading causes of neonatal sepsis and meningitis in the United States and have a worldwide distribution (1, 31). The C proteins of GBS, first recognized by Wilkinson (32), are present in approximately 60% of clinical isolates of GBS (17) and are immunogenic and protective against infection in animal models (21). These antigens, originally designated Ibc protein because of their presence on Ib and Ic (now Ia/c) strains of GBS, have never been fully characterized. Their size, number, structure, function, and virulence properties remain largely unknown. Other proteins present on certain strains of GBS include the R and X proteins, which have not been definitively shown to be protective antigenic determinants (22).

Several investigators have attempted to characterize the C-protein antigens of GBS. Techniques used to examine these antigens have included extraction into hot hydrochloric acid, detergent solubilization, and examination of proteins from the broth of growing GBS (3–5, 21, 28, 32). Proteins that are immunoreactive with polyclonal C-protein antisera have been reported in a range of 12 to 180 kDa. At least two C-protein antigens (designated alpha and beta) have been recognized by differential trypsin susceptibility and were shown to be expressed independently on different strains (5). Russell-Jones and colleagues (28, 29) reported that the beta-(trypsin-susceptible) antigen is a detergent-extractable protein with a molecular mass of 130 kDa and binds to the Fc region of human immunoglobulin A (IgA). Recently, two additional C-protein antigens (gamma and delta) have been proposed on the basis of serologic data (8). It has been suggested that C proteins may enhance virulence of GBS (26, 27).

Cleat and Timmis (13) reported the expression in *Escherichia coli* of recombinant GBS proteins that were immunoreactive with polyclonal antisera to alpha and beta C-protein antigens but did not demonstrate that these proteins were capable of eliciting protective immunity. Valtonen and colleagues (30) reported isolating a 14-kDa protein from the broth of a Ia/c strain of GBS that was capable of evoking mouse-protective antibodies. These researchers noted that antiserum raised against this polypeptide reacted with larger proteins extracted from GBS and speculated that the 14-kDa protein was a fragment of a larger protein.

Mutanolysin, a muralysin derived from *Streptomyces globisporus*, has been shown to cleave the cell wall of GBS and has been previously used for the purification of the polysaccharide capsule of this bacterium (11, 33). It appeared reasonable that mutanolysin could also be used to isolate surface-associated proteins from GBS under less harsh conditions and with greater preservation of native epitopes than with detergents or hot acid extraction. As the enzymatic extraction liberated a large number of proteins from GBS, only a subset of which were likely to be C proteins, we thought that monoclonal antibodies could assist in the recognition of individual proteins and might identify protective epitopes. In addition, monoclonal antibodies might reveal epitopes that are shared by or unique to individual C proteins. While monoclonal antibodies recognizing C proteins have previously been described (14, 28), any protective activity of these antibodies has not been reported.

We report a novel method for the extraction of proteins from the A909 (Ia/c) strain of GBS, using mutanolysin. We demonstrate that partially purified enzyme-extracted proteins are capable of eliciting antibodies protective against GBS infection in an animal model. A monoclonal antibody that recognizes a subset of these proteins induces in vitro opsonic killing of a C-protein-containing strain of GBS and confers protection in mice against lethal challenge with GBS, thus identifying a fully protective epitope of the GBS C-protein alpha-antigen.

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MATERIALS AND METHODS

Bacterial strains. The A909 (type Ia/$c_{\alpha,\beta}$), H36b (Ib/$c_{\alpha,\beta}$), and 090 (Ia) strains were originally obtained from Rebecca Lancefield. The 515 (Ia) strain of GBS is from the collection of the Channing Laboratory and was originally a clinical isolate.

Isolation of surface proteins from A909. The A909 strain of GBS was grown into stationary phase in 14 liters of dialysate of Columbia broth in a fermentor with the pH titrated to remain at 7.2. Bacterial cells were harvested, washed in phosphate-buffered saline (PBS), and resuspended in 1/20 the volume of 0.3 M potassium phosphate buffer–40% sucrose (pH 7.0); 560 U of mutanolysin (Sigma) was added and allowed to incubate overnight at 37°C with gentle stirring. Protoplasts were formed and removed by centrifugation. The supernatant was allowed to dialyze against deionized water, and a yellow-white precipitate formed. The precipitate was pelleted at 10,000 × *g* for 20 min and lyophilized. In order to characterize the antigens, further treatment of the extract with proteases was performed by the method of Wilkinson (32) as follows: digestion with trypsin (Sigma) (substrate/enzyme ratio = 4:1) in 50 mM Tris buffer (pH 8.0) for 2 h at 37°C. Digestion with hog gastric pepsin (Sigma) (substrate/enzyme ratio = 4:1) occurred in 50 mM citrate phosphate buffer (pH 2.5) for 2 h at 37°C.

Fractionation by column chromatography. The lyophilized powder was dissolved in 8 M urea. A solution of 5 mg/ml in 8 M urea–0.05 M Tris buffer (pH 7.4) was fractionated with a Sephacryl S-300 column (1.8 by 90 cm; Pharmacia) (16). Protein elution was monitored by A_{280}, and three peaks were noted. These were pooled, dialyzed against deionized water, and lyophilized.

Immunization of rabbits. New Zealand White rabbits were immunized with whole bacteria treated with Formalin as described by Lancefield et al. (21) or with the column fractions of the A909 surface extract emulsified in complete or incomplete Freund adjuvant or in normal saline according to the following schedule: day 1, 100 μg in complete Freund adjuvant subcutaneously; day 3, 100 μg intravenously; day 5, 100 μg intravenously; day 7, 100 μg in incomplete Freund adjuvant subcutaneously; day 14, 100 μg intramuscularly. Serum was obtained from the anesthetized rabbits at day 21 by cardiac puncture.

Mouse protection tests. The mouse protection method was adapted from Lancefield et al. (21). In brief, groups of 5 or 10 CD-1 outbred female mice (Charles River Laboratories, Wilmington, Mass.) of 6 to 8 weeks of age (approximately 25 g) were administered 1 ml of dilute antiserum or monoclonal antibody by intraperitoneal injection. Mice were challenged 24 h later with either 5×10^4 H36b (Ib/c) GBS or 5×10^8 515 (Ia) GBS. These inocula were determined to be the 90% lethal dose (LD_{90}) for these strains in unimmunized mice. Survivors were counted daily for 5 days.

SDS-PAGE and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (19). Gels had a 10 to 20% linear gradient of polyacrylamide, and electrophoresis was performed with a Hoeffer Mighty Small apparatus. Protein bands were visualized with Coomassie brilliant blue dye. Alternatively, electroblotting to nitrocellulose was performed by using 25 mM sodium phosphate (pH 6.5) and carried out either overnight at 125 mA or for 3 h at 300 mA. After being blocked with 5% skim milk in PBS containing 0.5% Tween 20, the blots were allowed to react with antiserum at dilutions of 1:100 to 1:500 in the same buffer. After reaction with goat anti-rabbit or anti-mouse IgG-alkaline phosphatase conjugate diluted 1:500, bands were visualized by the method of Blake et al. (7), utilizing the indoxyl phosphate-Nitro Blue Tetrazolium reagent.

Monoclonal antibody production. BALB/c mice were immunized by intraperitoneal injection with Formalin-killed A909 GBS (approximately 5×10^8 bacteria) emulsified in complete Freund adjuvant. At 3 weeks, the mice received a booster injection of the same dose of antigen in incomplete Freund adjuvant and a final booster injection 3 weeks later with antigen suspended in normal saline. The spleens of immune mice were removed and passed through a fine wire mesh to achieve a suspension of splenocytes. These were mixed with cells of the P3X63-AG8.653 nonsecreting mouse myeloma cell line at a ratio of 5:1, and fusion was induced with 40% polyethylene glycol at 37°C by the technique of Oi and Herzenberg (25). Fused cells were plated into 96-well plates at a density of 10^6 cells per well in RPMI 1640 (Gibco) with 15% fetal bovine serum. Fused cells were selected with hypoxanthineaminopterin-thymidine (HAT).

Detection of positive clones. An enzyme immunoassay for detection of relevant antibody-producing clones was developed. GBS surface protein extract was dissolved in 40 mM phosphate buffer (pH 7.0) at a concentration of 10 μg/ml and allowed to coat polystyrene 96-well plates for 2 h at 37°C. The wells were blocked with 5% skim milk in 40 mM phosphate buffer (pH 7.0) with 0.5% Tween 20. Hybrid cell supernatants were added directly to the wells and allowed to react for 1 h at 37°C. The plates were washed with 40 mM phosphate–0.5% Tween 20, and then a solution of alkaline phosphatase-conjugated anti-mouse IgG was added and incubated at 37°C. Color development was achieved with the phosphatase substrate, *p*-nitrophenyl phosphate (Sigma), and read at 405 nm by an automated enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech). Positive controls included immune mouse serum and negative controls included RPMI medium with fetal bovine serum.

Wells that reacted by enzyme immunoassay were further screened by Western blotting. Positive wells were expanded and cloned by limiting dilution. Ascitic fluid produced in pristane-primed BALB/c mice was diluted 1:1 in PBS and applied to a protein A-Sepharose column (Pierce). The column was washed with 40 column volumes of PBS, and IgG was eluted with 0.1 M glycine buffer at pH 2.8. Monoclonal antibody class, subclass, and light-chain type were determined by the above enzyme immunoassay, using alkaline phosphatase-conjugated goat antibodies to mouse heavy and light chains (Southern Biotechnology).

Opsonophagocytic assay. Opsonizing antibodies to C proteins of GBS were detected by an opsonophagocytic assay developed by Baltimore et al. (2) as an in vitro correlate to in vivo killing of GBS. In brief, 300 μl of human polymorphonuclear leukocytes (approximately 3×10^6) were mixed with GBS strain A909 (approx 1.5×10^6 CFU), absorbed human complement, and 100 μl of antibody preparation (peak 2 from a protein A-agarose affinity column). GBS cells were enumerated on blood and tryptic soy agar plates at $t = 0$ min and at $t = 60$ min. The result is reported as the "log kill," which is the difference in log CFU at 0 and 60 min in the presence of antibody and a complement source minus the difference in log CFU at 0 and 60 min in the presence of a complement source without antibody.

Detection of GBS beta-antigen. The organisms from one agar plate containing A909 or a control strain of GBS were placed in 0.5 ml of distilled H_2O and vortexed. A 100-μl aliquot of this suspension was mixed with 50 μl of an

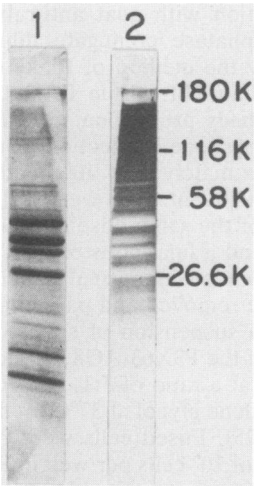

FIG. 1. Surface proteins of GBS analyzed by SDS-PAGE and Western blotting. Lane 1, SDS-PAGE of mutanolysin-extracted proteins from the A909 type Ia/c strain of GBS stained with Coomassie brilliant blue R250, demonstrating the numerous proteins extracted by this method. Lane 2, Western blot of the A909 surface extract with antiserum raised against a Ib/c strain of GBS at a 1:100 dilution, demonstrating the numerous immunoreactive proteins. The migrations of molecular mass standards (Sigma) are shown on the right. K, Kilodalton.

SDS-containing sample buffer and placed in a boiling water bath for 3 min. SDS-PAGE was performed as described above. The beta-antigen could easily be recognized as a 130-kDa band after Coomassie brilliant blue staining of the gel. The identity of the antigen was confirmed by immunoblotting with human myeloma IgA1 (29). This was performed as Western blotting, except that human myeloma IgA1 (kindly provided by Andrew Plaut, Boston, Mass.) was used at a concentration of 30 μg/ml instead of the primary antibody, and goat anti-human IgA conjugated to alkaline phosphatase (Cappel) at a dilution of 1:1,000 was used for the second antibody.

Statistical analysis. Opsonophagocytic data were analyzed for statistical significance and standard error with Statworks Version 1.1 (Cricket Software, Philadelphia, Pa.) on an Apple Macintosh IIcx. Exact log-rank analysis of mouse survival was performed by using StatXact version 1.0 (Cytel Software, Cambridge, Mass.) on an IBM PC, using the algorithm of Mehta et al. (24).

RESULTS

Preparation of surface proteins of GBS using mutanolysin. Presumed surface proteins were isolated from strain A909 (type Ia/c) by means of mutanolysin extraction. Dialysis of this extract against water resulted in the formation of a precipitate. The final yield of insoluble material from 14 liters of broth was 136 mg; 15 μg of this extract was subjected to SDS-PAGE and analyzed by Western blotting with rabbit antiserum raised against whole cells (treated with Formalin) of strain H36B (type Ib/c) GBS. Numerous immunoreactive bands were detected ranging in molecular mass from 20 to 160 kDa (Fig. 1). The range of molecular masses represented by these surface proteins is comparable with the ranges reported in previous studies that used either detergents or acid extraction (28).

The extract from GBS was insoluble at a concentration of 1 mg/ml in deionized water, PBS, or 3% Triton X-100; however, it was soluble in either 8 M urea or 6 M guanidine. Gel filtration column chromatography was performed on a 1-mg/ml solution in 8 M urea buffered with 0.05 M Tris (pH 7.4) with the use of a Sephacryl S-300 (Pharmacia) column (1.8 by 90 cm) equilibrated with the same buffer. There were three peaks detected in A_{280} (A_I, A_{II}, and A_{III}) (Fig. 2). The peak fractions were pooled and dialyzed extensively against distilled H_2O, and the resultant slurries were lyophilized. The insoluble pools were suspended in a sample buffer containing Tris and SDS and were subjected to SDS-PAGE (Fig. 2). A_I contained proteins of diverse molecular mass and did not appear to represent substantial purification relative to the original preparation. Pool A_{II}, however, was enriched in proteins of approximately 30 to 80 kDa. Pool A_{III} was highly enriched in a single 14-kDa protein, as determined by SDS-PAGE (Fig. 2) and by two-dimensional gel electrophoresis, with isoelectric focusing in the first dimension and SDS-PAGE in the second dimension (results not shown).

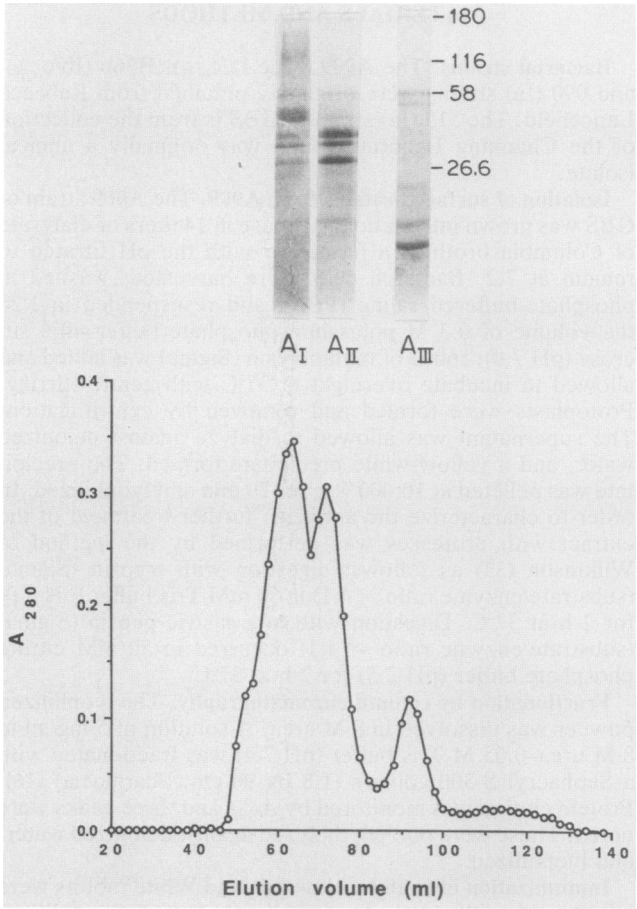

FIG. 2. Separation of mutanolysin surface extract of A909 GBS solubilized in 8 M urea on a Sephacryl S-300 column. Coomassie brilliant blue-stained SDS-PAGE of the pooled fractions A_I, A_{II}, and A_{III} are shown above the corresponding absorbance peak. The migrations of molecular mass standards (Sigma) are shown on the right (in kilodaltons).

Mouse protection by antisera to pools A_{II} and A_{III}. Because fraction A_I contained species of diverse molecular mass and did not appear to offer purification relative to the parent compound, this pool was not tested further. Rabbit antisera

TABLE 1. Protection of mice from lethal infection with H36b (Ib/c) GBS by using rabbit antiserum against the middle- and low-molecular-mass fraction of A909 (Ia/c) GBS surface protein extract[a]

Serum (dilution)	No. of mice surviving/total no. on day:				
	1	2	3	4	5
Anti-A_{II} (1:20)	4/5	4/5	4/5	4/5	4/5[b]
NRS[c] (1:20)	2/5	2/5	2/5	0/5	0/5
Anti-A_{III} (1:5)	5/5	5/5	5/5	5/5	5/5[d]
NRS (1:5)	5/5	4/5	3/5	1/5	1/5

[a] CD-1 outbred mice were passively immunized with rabbit antiserum to A_{II} and A_{III} and challenged at 24 h with LD_{90} of the heterologous strain. Controls received normal rabbit serum at the same dilution.

[b] $P = 0.04$ when anti-A_{II} and control serum at the same dilution are compared by exact log-rank test.

[c] NRS, Normal (preimmune) rabbit serum.

[d] $P = 0.008$ when anti-A_{III} and control serum at the same dilution are compared by exact log-rank test.

TABLE 2. In vitro opsonophagocytic killing of GBS strain A909 by monoclonal antibodies in the presence of human polymorphonuclear leukocytes and complement

Monoclonal antibody	Log_{10} killing with the following concn (μg/ml)[a]:			
	0.2	2.0	20.0	200.0
4G8	0.19 ± 0.14	0.48 ± 0.05	0.54 ± 0.17	0.54 ± 0.02
9BG5[b]	0.14 ± 0.06[c]	−0.05 ± 0.12[d]	0.10 ± 0.13[e]	−0.29 ± 0.08[f]

[a] Difference in log_{10} CFU after 1 h in the opsonophagocytic assay minus the difference in the absence of antibody. Values are the means and standard deviations of three experiments.

[b] 9BG5 is of the IgG2a type directed to a viral protein (10).

[c] Difference between 9BG5 and 4G8 values at the same antibody concentration is not statistically significant by Student's t test.

[d] $P = 0.02$ when 9BG5 and 4G8 values at the same antibody concentration are compared by Student's t test.

[e] $P = 0.024$ when 9BG5 and 4G8 values at the same antibody concentration are compared by Student's t test.

[f] $P < 0.001$ when 9BG5 and 4G8 values at the same antibody concentration are compared by Student's t test.

were raised against fractions A_{II} and A_{III}. The capacity of these antisera to protect from infection with a GBS strain of different capsular type but homologous for C protein was tested in a mouse protection assay, the method initially used by Lancefield et al. to define C proteins (21). The results of this assay are shown in Table 1 and demonstrate that the A_{II} antiserum was protective at a dilution of 1:20 and the A_{III} antiserum was protective at a dilution of 1:5. Although the A_{III} fraction was enriched for a single 14-kDa protein, antiserum raised to this fraction recognized a large number of bands of higher molecular mass in the mutanolysin extract (Fig. 3, lane 1). Antiserum to A_{II} also recognized a large number of bands (Fig. 3, lane 2).

Preparation of monoclonal antibodies. In order to study individual protein epitopes and to establish the possibility of shared epitopes in the proteins observed in the surface protein extract, monoclonal antibodies were developed. Monoclonal antibodies were prepared by using intact GBS strain A909 that had been treated with Formalin. Hybridoma clones were screened by ELISA, with the mutanolysin extract as antigen. One of these monoclonal antibodies, 4G8, was identified as an IgG2a kappa antibody and was chosen for further study because of the pattern observed in Western blots against the surface protein extract of the A909 strain of GBS (Fig. 3, lane 3). The antibody identified a series of regularly spaced bands ranging in apparent molecular mass from 30 to approximately 160 kDa at approximately 8-kDa intervals. Absorption of high-titered ascitic fluid containing 4G8 by the intact Ia/c strain A909 but not by the Ia strain 090 extensively removed reactive antibodies, as determined by ELISA. (Titer was reduced from $1:10^6$ to $<1:10^2$). Removal of the antibody by intact bacteria supports the surface localization of the epitope.

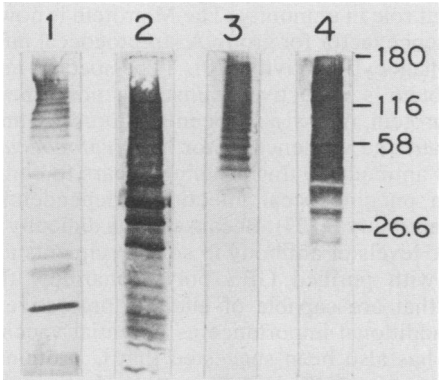

FIG. 3. Western blot of GBS A909 Ia/c surface protein extract with rabbit antiserum to the A_I (low-molecular-mass) pool (lane 1), with rabbit antiserum to the A_{II} (middle-molecular-mass) pool (lane 2), and with 4G8 monoclonal antibody (ascitic fluid diluted at 1:100) (lane 3), or with alpha-antigen-specific rabbit antiserum (kindly provided by L. Bevanger) (lane 4). The migrations of molecular mass standards (Sigma) are shown on the right (in kilodaltons).

In vitro and in vivo protective activity of the 4G8 monoclonal antibody. After purification of IgG from ascitic fluid with a protein A-Sepharose column, 4G8 antibody was tested for functional activity in an opsonophagocytic assay. Table 2 shows that the 4G8 antibody at concentrations of 2 to 200 μg/ml significantly reduced the number of CFUs of strain A909 GBS compared with an irrelevant monoclonal antibody (9BG5 directed against a viral antigen [10]) of the same class, subclass, and light-chain type and at the same concentrations. Killing was reduced at lower antibody concentration (0.2 μg/ml). Inactivation of the complement by heating to 56°C for 30 min eliminated killing by the 4G8 monoclonal antibody, thereby demonstrating that the killing is complement dependent (data not shown).

The 4G8 monoclonal antibody was then tested for its ability to protect mice against lethal infection with GBS. In pilot experiments, a dose-survival curve was established for the 4G8 antibody in the mouse protection assay, using the H36b strain (Ib/c) as the challenge strain. Enhanced survival was observed at doses as small as 10 μg per mouse when compared with mice receiving saline. To make certain that this effect was not due to the presence of nonspecific antibody, the effect of 100 μg of 4G8 antibody was compared with that of 100 μg of the irrelevant mouse monoclonal antibody (9BG5). A dose of 100 μg of 4G8 monoclonal antibody delivered intraperitoneally significantly enhanced the ability of mice to survive challenge with a C-protein-containing strain of GBS when compared with 9BG5 (Table 3).

Presence of the protective epitope on the alpha-antigen. Previous work on C proteins has distinguished between a trypsin-sensitive beta-antigen and a trypsin-resistant alpha-antigen (31). In order to determine whether the epitope recognized by the 4G8 was on the alpha- or beta-antigen, the

TABLE 3. Protection of mice from lethal infection with H36b (Ib/c) GBS by using the 4G8 monoclonal antibody[a]

Monoclonal antibody (μg)	No. of mice surviving/total no. on day:				
	1	2	3	4	5
4G8 (100)	10/10	10/10	9/10	9/10	9/10[b]
9BG5 (100)	10/10	7/10	5/10	4/10	4/10

[a] CD-1 outbred mice were passively immunized with 1 ml of PBS containing either 4G8 or 9BG5 mouse monoclonal antibody at 100 μg/ml and challenged at 24 h with LD_{90} GBS. The 9BG5 is of the IgG2a kappa type directed against a viral protein (10).

[b] $P = 0.03$ when 4G8 and 9BG5 are compared by exact log-rank test.

mutanolysin extract of GBS strain A909 was treated with pepsin or trypsin. Western blots were performed with these preparations and probed with the 4G8 monoclonal antibody (Fig. 4). The bands that react with the 4G8 antibody are susceptible to pepsin but not trypsin digestion. Bands of lower molecular mass also appeared on the Western blot after treatment with trypsin. This indicates that a portion of the protein is cleaved by trypsin but that antigenicity is retained after treatment. There were no immunoreactive bands seen after pepsin treatment, indicating that this treatment destroyed the epitope. These proteins are therefore consistent with the definition of the alpha-antigen of GBS C protein, which is distinguished from the beta-antigen by virtue of its resistance to trypsin degradation. In addition, monospecific antibody directed against the alpha-antigen (6) (kindly provided by Lars Bevanger, Trondheim, Norway) was used to immunoblot the mutanolysin extract and showed a stepladdering pattern of bands identical to that of the 4G8 antibody, further establishing this epitope on the alpha-antigen (Fig. 3, lane 4).

In order to show that the antigen recognized by the 4G8 monoclonal antibody was specific for the C-protein alpha-antigen, we sought to demonstrate that the antibody does not

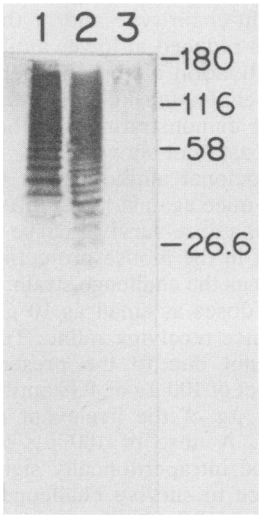

FIG. 4. Mutanolysin extract of the A909 strain of GBS untreated (lane 1) or processed with trypsin (lane 2) or pepsin (lane 3) at 37°C for 30 min prior to Western blotting with the 4G8 monoclonal antibody, demonstrating that the proteins recognized by the antibody are susceptible to pepsin but not to trypsin degradation. The migrations of molecular mass standards (Sigma) are shown on the right (in kilodaltons).

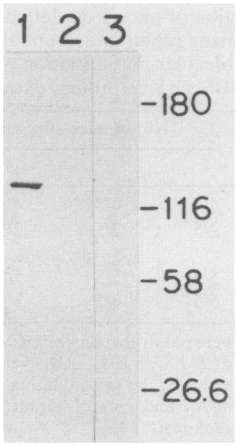

FIG. 5. Human myeloma IgA1 binding to the SDS-extracted beta-antigen of A909 type Ia/c strain of GBS. The blot is probed with human myeloma IgA1 and secondarily with goat anti-human IgA conjugated to alkaline phosphatase (lane 1) or with 4G8 monoclonal antibody and secondarily with goat anti-mouse IgG conjugated to alkaline phosphatase (lane 2). The type Ia 090 strain of GBS that does not contain C proteins is probed with myeloma IgA and shown as a negative control (lane 3). The migrations of molecular mass standards (Sigma) are shown on the right in kilodaltons.

bind to the C-protein beta-antigen. We used a modification of the method of Russell-Jones et al. (29) to extract the beta-antigen into detergent (SDS in this case). An extract from the A909 strain contained a band with a molecular mass of 130 kDa, easily visible on Coomassie brilliant blue-stained gel, which was absent in the control Ia strain 090. The identity of the antigen was confirmed by immunoblotting with human myeloma IgA (Fig. 5). There was no reaction between this preparation and the 4G8 monoclonal antibody in immunoblots (Fig. 5), indicating that this monoclonal antibody does not recognize beta-antigen in this preparation.

DISCUSSION

Virulence of and immunity to the group B streptococci has traditionally been addressed in terms of the well-described capsular polysaccharide (18). In other streptococci, especially group A streptococci, surface proteins appear to play a dominant role in immunity. The M protein is now known to be a virulence factor for group A streptococcal infection and has antiphagocytic activity (20). Type-specific immunity to the M protein is protective against infection. Pneumococcal surface protein A has also been explored as an antigenic determinant and virulence factor in *Streptococcus pneumoniae*, and antibody to this protein appears to confer protection from pneumococcal infection independently of anti-capsular antibody (9, 23). Because of the difficulty in eliciting protective levels of antibody in some recipients after immunization with purified GBS polysaccharides, the protein antigens that are capable of eliciting protective immunity take on additional importance as potential vaccine components. It has also been suggested that C proteins are virulence factors for GBS infection: type II strains that are C protein positive appear to evade in vitro opsonization (26) and phagocytosis (27) better than strains that lack C protein. C-protein-positive strains have also been shown to predominate in early neonatal infection (12).

In this study, proteins were enzymatically extracted from the A909 strain of GBS and shown to contain protective

antigens; these proteins could be used to immunize rabbits, and these antisera were protective against experimental infection with a strain of heterologous capsule type. Thus this extract contains, by definition, C proteins (i.e., seroreactive with C-protein antiserum and capable of eliciting protective immunity). These findings represent the first time that C proteins have been enzymatically extracted from the surface of GBS and shown to confer protective immunity. A monoclonal antibody which recognizes a trypsin-resistant, pepsin-susceptible group of these proteins induces killing in an opsonophagocytic assay and protects against lethal infection in a mouse model. These results indicate that the proteins recognized by the monoclonal antibody are C proteins. Earlier work by Bevanger and Naess (6) demonstrated that an alpha-antigen-specific antiserum delayed, but did not prevent, death in mice challenged with GBS. They found partial protection with this antiserum; however by day 5, 78% of the challenged mice died. Monoclonal antibody 4G8 protected 90% of the mice at 5 days. These differences may be due to differences in antibody titer, assay conditions, or epitope specificity between the polyclonal serum and the monoclonal antibody. It is interesting to note that in the opsonophagocytic assay, increases in the concentration of 4G8 above 2 μg/ml did not appear to increase the killing of the GBS. This may represent saturation of the antibody-binding sites on the bacteria.

Since multiple bands are recognized by the 4G8 monoclonal antibody, each band must contain an identical epitope. This is consistent with the possibility that the multiple bands seen on Western blots represent fragments of a larger protein. The M protein of group A streptococci has been shown to contain a region that is variably repeated within the same organism (15). The IgA protease of *Streptococcus sanguis* has recently been shown to contain a 20-amino-acid sequence which is repeated 10 times in the protein (14a). The appearance of regularly spaced bands on Western blots with monoclonal antibody 4G8 leads us to speculate that the GBS C-protein alpha-antigen contains a repeating domain that is either variably expressed or variably degraded, perhaps by proteolysis, thus accounting for the variation seen in molecular mass. An alternative explanation would be that the protein is covalently attached to some other regularly repeating structure, such as capsule or peptidoglycan. We find this latter possibility less likely, since such glycoproteins have not been described in similar bacteria.

The bands recognized by the 4G8 monoclonal antibody are resistant to trypsin digestion but susceptible to pepsin. This finding establishes that they are indeed proteins and that they represent the alpha-antigen or trypsin-resistant antigen described initially by Wilkinson (32). Previous attempts to isolate this antigen have yielded confusing results. Russell-Jones and Gotschlich described a heterogeneous collection of proteins extracted by detergent from the A909 strain of GBS (ranging in molecular mass from 20 to 120 kDa) that immunoreacted with both polyclonal antiserum and a monoclonal antibody. The protective nature of these antigens was not explored. The researchers speculated that the multiple bands represented the breakdown product of a larger parent. Cleat and Timmis (13) reported the expression in *E. coli* of a protein from cloned GBS genomic DNA that immunoreacted with polyclonal antiserum to the alpha component. This protein was described as a diffuse band of approximately 190 kDa by SDS-PAGE. Again, the protective nature of this protein was not explored. In contrast, the trypsin-susceptible or beta-antigen appears to exist as a discrete 130-kDa protein that binds to human IgA and does not react with the 4G8 antibody.

Monoclonal antibody 4G8 directed to a protective C-protein epitope should serve as a valuable tool for further research. The function of the C-protein alpha-antigen remains unknown. This monoclonal antibody will allow the substructure of the alpha-antigen to be explored and this antigenically important domain to be distinguished from other domains, such as structural regions or areas that are not surface exposed. The antibody may prove useful in attempts to detect variant proteins that immunoreact with polyclonal sera but fail to be recognized by the monoclonal antibody. The antibody may also allow the cloning and expression of the protective region of the GBS C-protein alpha-antigen.

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