INFECTION AND IMMUNITY, Aug. 1991, p. 2638–2644
0019-9567/91/082638-07$02.00/0

Vol. 59, No. 8

Phenotypic Diversity in the Alpha C Protein of Group B Streptococci

LAWRENCE C. MADOFF,[1,2,3]* SEIJI HORI,[1,3]† JAMES L. MICHEL,[1,3] CAROL J. BAKER,[4] AND DENNIS L. KASPER[1,2,3]

The Channing Laboratory, Brigham and Women's Hospital, 180 Longwood Avenue,[1] Division of Infectious Diseases, Beth Israel Hospital,[2] and Department of Medicine, Harvard Medical School,[3] Boston, Massachusetts 02115, and Departments of Pediatrics and Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030[4]

Received 14 March 1991/Accepted 16 May 1991

Group B streptococci (GBS) is the leading cause of neonatal sepsis and meningitis. C proteins are an immunologically important group of surface-associated antigens in GBS that remain incompletely characterized. Two C proteins have been designated alpha and beta on the basis of protease susceptibility. We recently used a monoclonal antibody to describe a protective epitope of the GBS alpha (or trypsin-resistant) C protein in the prototype Ia/c GBS strain. In the present study, we examined 51 GBS isolates for expression of C-protein alpha and beta antigens. The alpha antigen, as detected with monoclonal antibody in sodium dodecyl sulfate (SDS) extracts, appears as a heterogeneous series of proteins spaced 8 kDa apart on SDS-polyacrylamide gel electrophoresis, but has a maximum molecular mass that varies among strains from 62.5 to 167 kDa. By immunoblotting with human immunoglobulin A, polyclonal antiserum, or monoclonal antibody, the beta antigen, in contrast, appears as a single protein of molecular mass between 124 and 134 kDa. The amount of alpha antigen expressed by each strain was quantified by enzyme immunoassay inhibition and was found to vary markedly from strain to strain. The susceptibility of strains of GBS to opsonization and killing by human polymorphonuclear leukocytes in the presence of either complement alone or complement with alpha-specific monoclonal antibody was examined. Strains expressing the alpha antigen were less readily killed in the absence of specific antibody than were alpha-negative strains. Killing in the presence of alpha-specific monoclonal antibody was found to correlate directly with the maximum molecular mass of the alpha antigen and with the quantity of antigen on the bacterial cell surface. Isolates of GBS that express the alpha C protein vary widely in the quantity and molecular mass of the alpha antigen produced, and this heterogeneity appears to have biologic importance.

Group B streptococci (GBS) is the leading cause of neonatal sepsis in the United States and appears to be a growing problem worldwide (1, 27). Immunity to GBS has traditionally been considered to be directed against the well-described type-specific capsular polysaccharides (17). Another group of antigens, known collectively as C proteins, is also known to induce protective immunity in experimental animals (16). We recently explored the alpha (or trypsin-resistant) C protein by using a protective monoclonal antibody (18). This antigen appeared in Western blots (immunoblots) of the prototype Ia/c GBS strain A909 as a "ladderlike" series of bands with regularly spaced intervals, suggesting a repeating subunit structure (18). In addition, we expressed the GBS genes encoding the protective domains of the alpha and beta C proteins of the same GBS strain in *Escherichia coli* and demonstrated the ability of these recombinant proteins to elicit protective antibodies in the absence of other GBS determinants (19). Thus, the C-protein alpha antigen of GBS has been characterized at the molecular level in the prototype strain (A909; type Ia/c) (18), but the presence of this antigen in other strains has only been defined serologically, using polyclonal rabbit antiserum (5, 11).

Variability of serologically recognized antigens has been described between or even within strains of other bacterial pathogens of humans. For example, group A streptococci express a large variety of M-protein types (14). Differences between isolates in the molecular mass of pneumococcal surface protein A have been described recently (28). The C-protein beta antigen of GBS, when examined following acid extraction or secretion into broth, has been reported to vary in both molecular mass and functional immunoglobulin A (IgA)-binding activity (9). The importance to virulence and immunity of this type of strain-to-strain variability is not known. However, an increased quantity of capsule produced by type III GBS strains has been shown to confer resistance to opsonophagocytic killing (29) and to increase virulence in an animal model (12).

Strains of GBS possessing C proteins have been shown to have enhanced resistance to opsonophagocytosis (3, 21) and killing (22) in vitro, and the C proteins have therefore been postulated to have virulence properties. Clinical support for this role comes from the observation that C-protein-positive strains predominate in early-onset neonatal sepsis (10). The beta C protein binds to the Fc region of human IgA (25). This represents a possible mechanism by which it could affect virulence. No such biological function has been ascribed to the alpha C protein; however, recent work has suggested decreased complement binding by strains expressing the C-protein alpha antigen (23). To assess the role of the C proteins in virulence and immunity further, we decided to examine a larger number of isolates for expression of alpha C protein and to study their ability to resist opsonophagocytic killing in the presence and absence of a protective, alpha-specific, monoclonal antibody.

* Corresponding author.

† Present address: 2nd Department of Internal Medicine, Jikei University School of Medicine, 3-25-8 Nishi-Shinbashi, Minato-ku, Tokyo 105, Japan.

We report the detection of the alpha and beta C proteins in clinical isolates by Western blot, using specific antisera and monoclonal antibodies. We define the existence of the alpha C protein on a variety of strains, using an alpha-specific and protective monoclonal antibody (4G8). We demonstrate that the expression of the alpha C-protein antigen varies between strains when assessed either qualitatively by Western blot or quantitatively by enzyme-linked immunoassay (ELISA) inhibition. Compared with alpha C-protein-negative strains, alpha C-protein-positive isolates resist opsonophagocytic killing in the absence of antibody. The potential biologic significance of the observed antigenic diversity is demonstrated by the correlation of the quantity of alpha antigen expressed, as well as its maximum molecular mass, with the extent of in vitro opsonophagocytic killing by monoclonal antibody.

MATERIALS AND METHODS

Bacterial strains. Nineteen strains of GBS were from the collection of the Channing Laboratory and were originally clinical isolates. These included the prototype strains 090 (Ia), A909 (Ia/c), H36B (Ib/c), and 18RS21 (II), which were obtained from the late Rebecca Lancefield. Thirty-two strains were clinical isolates from hospitals associated with the Baylor College of Medicine, predominantly from cases of sepsis and meningitis. Serotyping was performed by capillary precipitation with hyperimmune rabbit antiserum after the method of Lancefield et al. (16).

Preparation of antisera. C-protein antiserum was prepared by the method of Lancefield et al. (16). Briefly, New Zealand White rabbits were immunized intravenously with 0.5 ml of formalin-killed A909 (type Ia/c) GBS for 3 days and then with 1.0 ml for an additional 5 to 9 days. After seven doses, rabbits were bled daily to determine the reaction of sera with extracts from the prototype Ia, Ib/c, and Ia/c strains. Once C-protein specificity was observed (i.e., there was reactivity only with the Ib/c and Ia/c extracts), rabbits were exsanguinated, and sera were separated from whole blood and stored at −20°C. Antiserum to *E. coli* carrying the pJMS1 plasmid and expressing the GBS beta-antigen protective epitope was prepared as described previously (19). In brief, rabbits were immunized by intravenous injection of a whole-cell sonic extract of *E. coli* pJMS1 three times weekly for 3 weeks. Monoclonal antibody 4G8 was developed as described previously (18) by standard techniques from a mouse immunized with intact formalinized GBS of strain A909 and identified by ELISA. Serotyping of GBS strains was performed by using capillary precipitation (15).

ELISA. An ELISA inhibition technique was developed to quantify the amount of alpha antigen present on the surface of intact GBS. The assay was based on inhibition by intact GBS of the binding of a specific monoclonal antibody recognizing the C-protein alpha antigen [4G8; mouse IgG2a(κ) directed to a protective epitope of the C-protein alpha antigen] (18) to a plate coated with surface proteins from the A909 (Ia/c) strain of GBS. Polystyrene ELISA plates were prepared by coating with a 10-μg/ml solution of mutanolysin-extracted surface proteins from the A909 (Ia/c) strain of GBS in 40 mM phosphate buffer (pH 7.0) for 1 h at 37°C. GBS strains were grown to exponential phase (A_{650}, 0.3) in Todd-Hewitt broth (GIBCO). Bacteria were washed by centrifugation in phosphate-buffered saline (PBS) and resuspended in 1/10 of the original volume of 40 mM phosphate buffer (pH 7.0) with 0.5% Tween 20. The bacterial suspension was then serially diluted in a 96-well plate before addition of an equal volume of a 1:10,000 dilution of ascites containing the 4G8 monoclonal antibody in the same buffer. The plates were then incubated at 37°C for 1 h on a plate shaker to allow absorption of antibody. The samples were transferred to the coated ELISA plates and allowed to incubate for 1 h at 37°C. The plates were washed with 40 mM phosphate–0.5% Tween 20; then a solution of alkaline phosphatase-conjugated anti-mouse IgG was added, and plates were incubated at 37°C for 2 h. The phosphatase substrate *p*-nitrophenyl phosphate (Sigma Chemical Co.) was added, and the A_{405} was obtained by automated ELISA plate reader (Dynatech, Chantilly, Va.). The A_{405} at each bacterial concentration was divided by the A_{405} of an uninhibited control to obtain percent ELISA reactivity. This percentage was plotted against bacterial concentration to calculate a 50% inhibitory concentration (IC_{50}) for each strain.

Preparation of beta-antigen-specific monoclonal antibody. Beta antigen was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on single-well 8% gels (5.0 by 8.0 by 0.015 cm). A suspension of A909 bacteria (overnight growth of a blood agar plate suspended in 0.5 ml of H_2O) was extracted by boiling for 3 min in SDS-PAGE sample buffer. A distinct band was visualized at 130 kDa by Coomassie blue staining of a parallel lane. The 130-kDa band was cut from the gel, finely chopped, and injected intraperitoneally into BALB/c mice. Booster vaccinations were performed at 3 and 6 weeks. The spleens of immune mice were removed and passed through a fine wire mesh to achieve a suspension of splenocytes. These cells were mixed with cells of the P3X63-AG8.653 nonsecreting mouse myeloma line at a ratio of 5:1, and fusion was induced with 40% polyethylene glycol at 37°C after the technique of Oi and Herzenberg (20). 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 hypoxanthine-aminopterin-thymidine. Hybridoma supernatants were screened for the presence of specific antibody by ELISA; polystyrene plates coated with the Ib/c (alpha negative, beta positive) strain 7357b (10^8 CFU/ml in 40 mM phosphate buffer, pH 7.0, at 37°C for 1 h) were used. Cell culture well supernatants were transferred to the ELISA plates, which were incubated for 1 h and probed with alkaline phosphatase-conjugated goat anti-mouse IgG (Tago, Burlingame, Calif.) as described above; the A_{405} was read in an automated ELISA plate reader. Reactive wells were screened further by Western blot. A total of three wells from two successive fusions were reactive by Western blot with the 130-kDa beta C protein. One of these, 3E7, was successfully cloned by limiting dilution (20) and identified as an IgG2b(κ) by reactivity with subclass-specific antiserum (Southern Biotechnology Associates, Birmingham, Ala.).

Immunoblotting of GBS extracts. Immunoblotting of SDS extracts from the bacterial strains with monoclonal antibody was performed as follows: bacteria from the surface of a blood agar plate containing the GBS strain were removed with a sterile cotton-tipped swab and suspended in PBS until a concentration giving an A_{650} of 0.3 was obtained. A 100-μl aliquot of the suspension was placed in a small plastic conical tube, and a 50-μl volume of 187 mM Tris buffer (pH 6.8) with 15% SDS, 30% glycerol, and 0.006% bromphenol blue was added. The suspension was placed in a boiling water bath for 3 min and then centrifuged at 10,000 × *g* for 2 min. A 15-μl portion of the supernatant was applied to an 8% polyacrylamide gel, and electrophoresis was performed by the technique of Laemmli (13). Electroblotting onto

nitrocellulose sheets was performed with a semidry blotting apparatus (Hoeffer Instruments, San Francisco, Calif.). Previous studies showed that bands immunoreactive with the 4G8 antibody did not transfer with the use of 25 mM phosphate (pH 6.5) transfer buffer (18), so 20 mM Tris–150 mM glycine buffer (pH 8.0) with 20% methanol (26) was used instead. After sheets were blocked with PBS containing 0.5% Tween 20 and 5% skim milk for 2 h at 37°C, the nitrocellulose was probed with one of the following: (i) a 1:1,000 dilution of ascites containing 4G8 antibody, (ii) a 1:1,000 dilution of beta-specific rabbit antiserum (raised to *E. coli* clone pJMS1) expressing beta antigen (19), or (iii) human myeloma IgA1 (kindly provided by A. Plaut and A. Wright, Boston, Mass.) at a concentration of 10 μg/ml. The nitrocellulose was then washed and probed with antibody to mouse or rabbit IgG or human IgA conjugated to alkaline phosphatase (Tago), and immunoreactive bands were visualized utilizing the indoxyl phosphate-nitroblue tetrazolium reagent (8). The migrations of prestained molecular weight standards (Sigma) were used to calculate the molecular weights of the immunoreactive bands.

Opsonophagocytic assay. The functional capacity of antibodies to C proteins of GBS was assessed by an opsonophagocytic assay (4) measuring in vitro killing of GBS. In brief, a 300-μl volume of human polymorphonuclear leukocytes (approximately 3×10^6 cells) was mixed with the test GBS strain (approximately 1.5×10^6 CFU), 50 μl of human serum (as a complement source), and 100 μl of the 4G8 antibody preparation (purified by affinity chromatography on a protein A-agarose column) diluted 1:100. Viable GBS cells were enumerated as 10-fold dilutions on blood agar plates immediately and at 60 min. The result is reported as the "log kill," which is the difference between killing with monoclonal antibody 4G8 and without this antibody. Human serum was prepared for use as a complement source by absorption on ice for 30 min, with the contents of one blood agar plate containing the GBS serotype of the strain assayed. The bacteria were removed by centrifugation, and the absorbed serum was stored in aliquots at −80°C.

Statistics. Correlation coefficients were calculated with Cricket Graph version 1.3 (Cricket Software, Malvern, Pa.), and Student's *t* tests were performed with Statview SE (Abacus Concepts, Berkeley, Calif.) on an Apple Macintosh IIcx.

RESULTS

Immunoblotting of GBS extracts with 4G8 alpha-specific monoclonal antibody. Clinical isolates of GBS were extracted as described above and immunoblotted with the 4G8 monoclonal antibody. Of the 51 isolates studied, 37 showed immunoreactive protein bands, all of which had the characteristic ladder pattern described previously for the alpha antigen (18). However, the molecular mass of the largest protein varied between strains (examples shown in Fig. 1). The calculated molecular mass of the largest protein seen in these GBS strains varied from 62.5 to 167 kDa. The spacing between the multiple bands in each strain appeared fixed at 8 kDa, and the molecular weight of the lower-molecular-weight bands appeared to be identical between strains. In every isolate the highest-molecular-weight band stained darkest on immunoblot, with the smaller bands appearing lighter.

To examine the effect of growth phase on the alpha C-protein phenotype, four isolates were examined during early, exponential, and stationary phases of growth. All

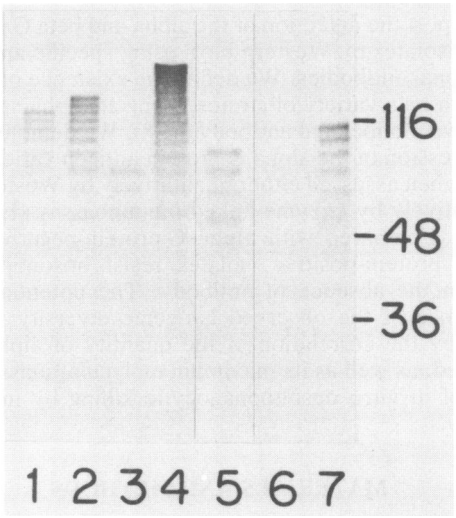

FIG. 1. Western blots of SDS extracts from representative GBS strains probed with 4G8 monoclonal antibody. The banding pattern demonstrates the variability of the molecular mass of the largest band and the conserved stepladdering appearance. Lanes: 1, S17; 2, S18; 3, S19; 4, S20; 5, S21; 6, prototype Ia strain 090; 7, prototype Ia/c strain A909. Molecular weight standards (10^3) are marked on the right.

were found to have identical patterns of immunoreactivity regardless of the stage of growth (data not shown). The molecular masses of the bands remained constant for each strain in duplicate experiments. There was no relationship between the capsular type of the GBS strain and the molecular masses of the alpha antigen proteins on immunoblot. Two strains containing the Ia polysaccharide (as determined by capillary precipitation) showed weakly reactive bands by immunoblot even though they had failed to react with C-protein typing antiserum in capillary precipitation.

ELISA inhibition with GBS strains. The relative quantitative surface expression of C-protein alpha antigen was determined by measuring the ability of intact, viable bacteria grown to a standardized concentration to inhibit the binding of 4G8 monoclonal antibody to mutanolysin-extracted surface proteins in the ELISA. Representative ELISA inhibition curves are shown in Fig. 2. Strains of GBS were able to inhibit the ELISA reactivity of 4G8 in an inoculum-dependent fashion. The number of CFU required to inhibit 50% of color development was determined and expressed as the IC_{50} for the 37 alpha-positive strains. The IC_{50} values ranged from 1.57×10^9 to 1.95×10^7 CFU/ml, representing approximately an 80-fold difference in surface expression of alpha antigen between isolates containing this antigen. The reciprocal of the IC_{50} thus increases directly with the quantity of alpha C protein as determined by ELISA inhibition. Serotype Ia strain 090, as well as three type III strains believed not to contain C proteins, failed to inhibit ELISA reactivity. Seventeen strains failed to inhibit 4G8 antibody at all, even at the highest bacterial concentration (approximately 10^{10} CFU/ml). The two strains which were serotyped by capillary precipitin as Ia and showed immunoreactive bands by immunoblot also showed partial inhibition in the ELISA, demonstrating that they were, in fact, Ia/c (alpha) strains. Among strains that showed inhibition, there appeared to be no relationship between capsular type and IC_{50}. Data on the time required to visualize capillary precipitation were available for 19 alpha C-protein-containing strains. This time

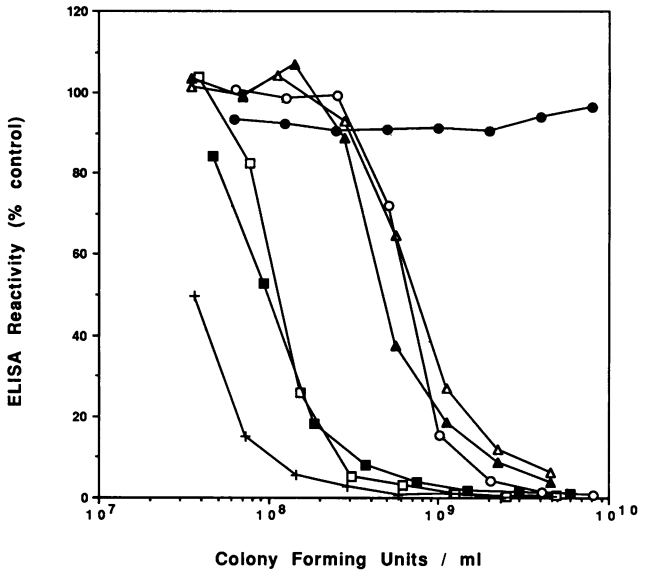

FIG. 2. ELISA inhibition curves for representative strains of GBS. The IC_{50} for each strain was determined graphically from the intersection of the ELISA inhibition curve and the 50% inhibition line. The same strains are represented as in Fig. 1. ○, S17; ■, S18; △, S19; +, S20; ▲, S21; ●, prototype Ia strain 090; □, prototype Ia/c strain A909.

correlated inversely with the quantity of alpha C protein (reciprocal IC_{50}; $r = -0.6$; $P < 0.01$); thus, an increased quantity of alpha antigen was related to a decreased time required to see capillary precipitation, using standard Lancefield typing antiserum.

Correlation between antibody-mediated opsonophagocytosis and expression of alpha antigen. To test the functional significance of the variability in alpha-antigen expression, monoclonal antibody 4G8-mediated killing of the 51 strains was tested in an in vitro opsonophagocytic assay. Among the alpha-positive strains, the log kill ranged from −0.205 to 1.63. The log kill by monoclonal antibody 4G8 (Table 1) was significantly greater in the strains expressing alpha antigen than in those lacking alpha antigen ($P = 0.0001$). Furthermore, this effect was related to the quantitative expression of the alpha antigen as measured by IC_{50} in the ELISA. The log kill in the presence of monoclonal antibody correlated significantly and directly with the reciprocal IC_{50} (which increases with increasing quantity of alpha antigen) of 37 strains expressing alpha antigen (Fig. 3A; $r = 0.52$; $P < 0.01$). In addition, the log kill correlated directly with the molecular weight of the highest band seen in immunoblot (Fig. 3B; $r = 0.51$; $P < 0.01$). This indicates that increased expression of the alpha C protein, or an increase in the molecular weight of the antigen, renders the bacteria more susceptible to antibody-mediated opsonic killing.

Since the quantity of alpha antigen as determined by ELISA inhibition (reciprocal IC_{50}) and the maximum molecular weight were both significantly correlated with log kill in the opsonic assay, we tested the correlation between these two independently assayed variables. The significant correlation between these two variables indicated that ELISA inhibition increased with the molecular weight of the largest band seen in immunoblot (Fig. 3C; $r = 0.8$; $P < 0.01$).

Expression of alpha C protein and antibody-independent opsonophagocytic killing. To test whether the C-protein alpha antigen conferred protection against host defenses, killing of GBS by polymorphonuclear leukocytes in the presence of complement but without specific antibody was studied. Complement-mediated killing (i.e., in the presence of human serum complement but in the absence of monoclonal antibody) was greater for the alpha-negative isolates (14 alpha-negative strains: log kill = 1.18 ± 0.76; 37 alpha-positive strains: log kill = 0.75 ± 0.57; $P = 0.03$). Therefore, the alpha C protein conferred relative protection against complement-mediated opsonophagocytic killing in the absence of specific antibody.

Immunoblotting with beta-specific antibodies and myeloma human IgA. To characterize the isolates further, we determined the presence of the beta C protein in each strain by immunoblot. Because the beta C protein has been shown to bind human IgA (25), the SDS-solubilized proteins isolated from the GBS strains were immunoblotted with human myeloma IgA. The isolates were also examined with rabbit antiserum raised to the pJMS1 strain of *E. coli*, which expresses the beta-antigen protective epitope, and with mouse monoclonal antibody 3E7 to the native beta C protein. An immunoreactive band was detected in 17 of the 51 isolates at 124 to 134 kDa, with only slight variation of the molecular weight between strains (examples shown in Fig. 4). There was complete concordance between the methods used; all IgA-binding antigens also reacted with the pJMS1 antiserum and with 3E7 monoclonal antibody. Only a single immunoreactive band was detected in each strain. All strains that reacted by capillary precipitation with C-protein antiserum contained the alpha or beta antigen or both. The distribution of the alpha and beta C-protein antigens among the C-protein-positive strains is shown in Table 2.

TABLE 1. Comparison of alpha C-protein-positive and -negative strains of GBS

Capsule type	Alpha	No. of strains	Opsonic killing (log_{10}) by 4G8 antibody (mean ± SEM)[a]
Ia	Negative	2	−0.09 ± 0.01
	Positive	14	0.66 ± 0.014[b]
Ib	Negative	4	0.14 ± 0.07
	Positive	11	0.72 ± 0.14[c]
II	Negative	5	−0.19 ± 0.08
	Positive	12	0.14 ± 0.08[d]
III	Negative	3	−0.22 ± 0.17
	Positive	0	NA
Total, all types	Negative	14	−0.11 ± 0.06
	Positive	37	0.51 ± 0.08[e]

[a] In the presence of polymorphonuclear leukocytes and complement. NA, not applicable.
[b] P = not significant. Student's t test (unpaired) versus alpha negative of same serotype.
[c] $P = 0.01$.
[d] $P = 0.03$.
[e] $P = 0.0001$.

DISCUSSION

The alpha antigen of the GBS C protein has been defined by its protease susceptibility (30), its ability to elicit protective antibody (7, 16), and its distinctive laddering appearance on Western blots when probed with monospecific or monoclonal antibody (6, 18). While the beta (or trypsin-suscepti-

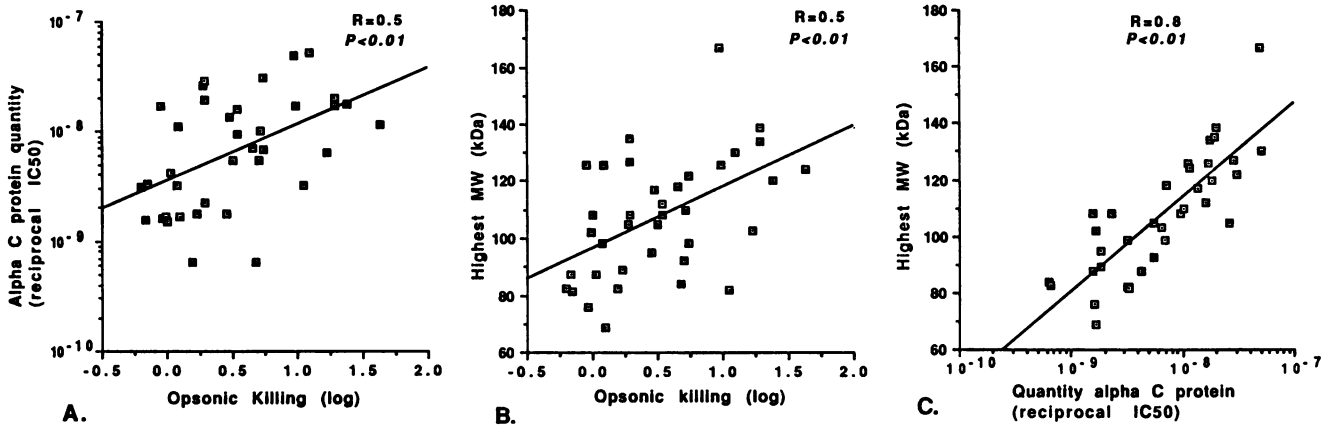

FIG. 3. (A) Log kill in the opsonophagocytic assay for 37 alpha-positive GBS strains versus reciprocal IC_{50} of alpha C protein (as determined by ELISA inhibition) showing the correlation between these two variables. (B) Opsonophagocytic killing versus highest molecular weight of alpha C-protein antigen (as determined by Western blot) showing the correlation between these two variables. (C) Quantity of alpha C protein (reciprocal IC_{50}) versus maximum molecular mass showing the correlation between these two variables. The best-fit line as determined by linear regression is shown for each set of points.

ble) C protein has been found to bind IgA (25), no function for the alpha C protein has yet been established. However, some data suggest that C proteins may confer virulence to GBS (3, 10, 21, 22). It has been demonstrated previously that both monoclonal antibody (18) and specific antiserum (7) directed to the alpha antigen are protective against experimental infection with GBS. In this study, we show that alpha-positive strains are more resistant to opsonization in the absence of antibody than alpha-negative strains. In the presence of alpha-specific monoclonal antibody, the opsonophagocytic killing of GBS strains is dependent on the quantity of antigen expressed and on the molecular weight of the largest immunoreactive band of alpha C protein. We also found that the magnitude of the opsonophagocytic killing reported for type II strains in this system was smaller than for the type Ia and Ib strains (Table 1). This difference is most likely due to the different type-specific capsular polysaccharide of these strains, but did not appear to be due to differences in the alpha C-protein antigen.

Our data indicate that the alpha and beta antigens can be detected by Western blots of SDS-extracted bacteria and that the detection of the alpha antigen in several strains thought previously to be C protein negative demonstrates that this technique may be more sensitive than immunoprecipitation methods. The highest molecular weight of the alpha antigen varies considerably between strains and appears to be a stable phenotypic trait of the strain that may be useful for identifying or classifying isolates.

We found considerable variation in the quantity of alpha antigen present, with an 80-fold difference between the lowest detectable positive strains and the highest-producing strains. The laddering pattern of the alpha antigen on immunoblot remained invariant between strains, with 8-kDa intervals between adjacent bands. However, the molecular mass of the largest band varied by >100 kDa between strains. In addition, the quantity of alpha antigen expressed by a given strain was directly related to the molecular mass of the largest immunoreactive protein band detected by Western blot analysis. These were stable phenotypic traits of each particular strain that were unaltered by laboratory passage.

There are several possible explanations for this correlation: (i) the higher-molecular-mass proteins possess a greater number of antibody binding sites (i.e., a repeating subunit), thus binding more antibody; (ii) the larger antigens have greater exposure on the surface of the intact bacteria (perhaps extending further through the other surface structures, such as capsular polysaccharide), or (iii) strains that produce larger alpha antigens also produce a greater number of antigen molecules per bacterium. Our data cannot distinguish between these possibilities, and it is possible, of course, that more than one of these hypotheses are correct.

The mechanism of variability in alpha-antigen expression at the molecular level also remains unknown. The gene encoding the alpha antigen might contain a variable number of copies of a repeating subunit. It is also possible that variability occurs at the transcriptional level through an as yet undescribed mechanism or that variation occurs posttranslationally perhaps through differential protease cleavage. Any of these possibilities could also account for the observed stepladdering seen on immunoblot. Preliminary studies employing Southern hybridization analysis with the cloned alpha-antigen gene from the prototype Ia/c strain probing genomic DNA of several GBS strains demonstrate polymorphism in or near the alpha C-protein gene (unpublished data).

In contrast to the alpha C protein, we found little variability among the beta C proteins expressed in the 17 strains that expressed this antigen. All of these strains expressed a single protein band of similar molecular mass which immunoreacted with both monoclonal and polyclonal antibodies to the beta C protein, and all were visualized by IgA immunoblot. Brady and Boyle (9) have reported heterogeneity in beta C-protein size and variability in the capacity to bind IgA among 18 clinical GBS isolates. These authors used hot HCl extraction of antigen rather than SDS extraction. They also examined secretion of protein into broth, whereas we examined washed bacteria directly. These methodologic differences may account for the different findings. For example, it is possible that the beta C-protein antigen is cleaved proteolytically after its secretion, thus yielding smaller subunits. It is possible that strain variation may account for the different findings since we examined different isolates. However, our results corroborate those of Russell-Jones et al. (24, 25), who also examined detergent extracts of GBS

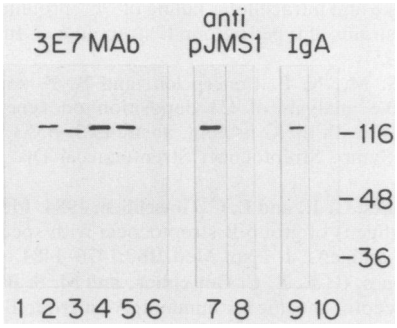

FIG. 4. Immunoblot from representative SDS extracts of GBS strains with monoclonal antibody (MAb) 3E7, with antibody to pJMS1 *E. coli* expressing the beta C protein, and with human myeloma IgA1. The migrations of molecular weight standards are shown on the right. Strain 090 (lanes 1, 8, and 10) is C protein negative, and strain A909 (lanes 2, 7, and 9) contains both the alpha and the beta C proteins. Lanes 3 to 6 are clinical isolates S12, S14, S15, and S16. S16 is alpha positive and beta negative. Molecular weight standards (10^3) are marked on the right.

strains. They found only a single beta C protein of 130-kDa molecular mass which immunoreacted with a monoclonal antibody and bound to human IgA.

The biological function of the C-protein alpha antigen and its role in virulence remain unknown. It has been suggested that this protein inhibits complement activity (23) and that C-protein-positive strains are better able to resist opsonophagocytosis (3, 21) and killing (22) than strains lacking C protein; we also observed this phenomenon. We did not detect a correlation between complement-mediated killing in the absence of antibody and the amount of alpha antigen expressed. However, such a correlation may be masked by other factors known to influence opsonophagocytic killing such as the amount of capsular polysaccharide. It is possible that any anticomplementary effect is not quantitatively related to the size or amount of alpha C protein, but merely to its presence. The domain responsible for complement resistance may be distinct from the domain that varies between strains. To establish with complete certainty that the observed differences in strains are due only to differences in the alpha C protein, it will be necessary to study isogenic strains which differ only in this gene.

The distribution of C-protein types alpha and beta among the isolates studied (Table 2) closely parallels that reported previously in clinical isolates (11). The vast majority (90% in this study) of C-protein-positive isolates contain the alpha C protein either alone or with the beta C protein. In contrast, only 42% of these isolates contain the beta C protein. This distribution suggests that the alpha C-protein antigen may represent the preferred target of immunotherapy for prevention of GBS infection. The use of a C protein conjugated to a capsular polysaccharide has been proposed (2) for active immunization against GBS, and the alpha C protein could be useful in this capacity.

TABLE 2. Distribution of alpha and beta antigens in C-protein-positive GBS isolates[a]

Serotype	*n*	No. (%) of isolates expressing indicated antigen(s)		
		Alpha only	Beta only	Alpha and beta
Ia/c	14	12 (86)	0 (0)	2 (14)
Ib/c	15	2 (13)	4 (27)	9 (60)
II/c	12	10 (83)	0 (0)	2 (17)
Total	41	24 (58)	4 (10)	13 (32)

[a] Antigen positivity as determined by immunoblot.

This study demonstrates a large qualitative and quantitative variability in the expression of the trypsin-resistant or alpha C-protein antigen in clinical isolates of GBS. Antibody-mediated opsonophagocytic killing of these strains in an in vitro assay varies with the expression of this antigen, which suggests that this variability may play a role in immunity to GBS infection. The mechanisms of this diversity and its potential role in the virulence of these bacteria are under study.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI23339, AI28500, and F32AI08110 from the National Institutes of Health and by the William Randolph Hearst Fund at Harvard Medical School. L.C.M. was supported by National Research Service Award T32AI07061 and Physician Scientist Award K11AI00981 from the National Institutes of Health. J.L.M. is a recipient of the Lederle Young Investigator Award in Vaccine Development of the Infectious Disease Society of America.

REFERENCES

1. **Baker, C. J.** 1986. Group B streptococcal infection in newborns. N. Engl. J. Med. **314**:1702–1704.
2. **Baker, C. J.** 1990. Immunization to prevent group B streptococcal disease: victories and vexations. J. Infect. Dis. **161**:917–921.
3. **Baker, C. J., B. J. Webb, D. L. Kasper, and M. S. Edwards.** 1986. The role of complement and antibody in opsonophagocytosis of type II group B streptococci. J. Infect. Dis. **154**:47–54.
4. **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**:673–678.
5. **Bevanger, L.** 1983. Ibc proteins as serotype markers of group B streptococci. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **91**:231–234.
6. **Bevanger, L., O. Iversen, J. A. Maeland, and A. I. Naess.** 1990. Characterization of the c-protein antigens of group B streptococci (GBS) using murine monoclonal antibodies, abstr. P77, p. 97. XI Lancefield Int. Symp. Streptococci Streptococcal Dis. 1990, Siena, Italy.
7. **Bevanger, L., and A. I. Naess.** 1985. Mouse-protective antibodies against the Ibc proteins of group B streptococci. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **93**:121–124.
8. **Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. Anal. Biochem. **136**:175–179.
9. **Brady, L. J., and M. D. P. Boyle.** 1989. Identification of non-immunoglobulin A-Fc-binding forms and low-molecular-weight secreted forms of the group B streptococcal β antigen. Infect. Immun. **57**:1573–1581.
10. **Chun, C., E. M. Ayoub, L. J. Brady, M. P. Boyle, and H. Dillon.** 1989. C protein of group B streptococci: role in virulence. Pediatr. Res. **25**:175A.
11. **Johnson, D. R., and P. Ferrieri.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J. Clin. Microbiol. **19**:506–510.
12. **Klegerman, M. E., K. M. Boyer, C. K. Papierniak, L. Levine, and S. P. Gotoff.** 1984. Type-specific capsular antigen is associated with virulence in late-onset group B streptococcal type III disease. Infect. Immun. **44**:124–129.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lancefield, R. C.** 1962. Current knowledge of type-specific M antigens of group A streptococci. J. Immunol. **89**:307–313.

15. **Lancefield, R. C.** 1975. Antigens of group B streptococci. Relation to mouse-protective antibodies and immunity, p. 145–151. *In* J. B. Robbins et al. (ed.), New approaches for inducing natural immunity to pyogenic organisms. National Institutes of Health, Bethesda, Md.
16. **Lancefield, R. C., M. McCarty, and W. N. Everly.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J. Exp. Med. **142**:165–179.
17. **Levy, N. J., A. Nicholson-Weller, C. J. Baker, and D. L. Kasper.** 1984. Potentiation of virulence by group B streptococcal polysaccharides. J. Infect. Dis. **149**:851–860.
18. **Madoff, L. C., J. L. Michel, and D. L. Kasper.** 1991. A monoclonal antibody identifies a protective C-protein alpha-antigen epitope in group B streptococci. Infect. Immun. **59**:204–210.
19. **Michel, J. L., L. C. Madoff, D. E. Kling, D. L. Kasper, and F. M. Ausubel.** 1991. Cloned alpha and beta C-protein antigens of group B streptococci elicit protective immunity. Infect. Immun. **59**:2023–2028.
20. **Oi, V. T., and L. A. Herzenberg.** 1980. Immunoglobulin-producing hybrid cell lines, p. 351–372. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman and Co., New York.
21. **Payne, N. R., and P. Ferrieri.** 1985. The relation of the Ibc protein antigen to the opsonization differences between strains of type II group B streptococci. J. Infect. Dis. **151**:672–681.
22. **Payne, N. R., Y. K. Kim, and P. Ferrieri.** 1987. Effect of differences in antibody and complement requirements on phagocytic uptake and intracellular killing of "c" protein-positive and -negative strains of type II group B streptococci. Infect. Immun. **55**:1243–1251.
23. **Puentes, S. M., N. F. Concepcion, and B. F. Anthony.** 1990. Quantitative analysis of C3 deposition on type II group B streptococci with the C protein, abstr. L73, p. 53. XIth Lancefield Int. Symp. Streptococci Streptococcal Dis. 1990, Siena, Italy.
24. **Russell-Jones, G. J., and E. C. Gotschlich.** 1984. Identification of protein antigens of group B streptococci with special reference to the Ibc antigens. J. Exp. Med. **160**:1476–1484.
25. **Russell-Jones, G. J., E. C. Gotschlich, and M. S. Blake.** 1984. A surface receptor specific for human IgA on group B streptococci possessing the Ibc protein antigen. J. Exp. Med. **160**:1467–1475.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
27. **Walsh, J., and S. Hutchins.** 1989. Group B streptococcal disease: its importance in the developing world and prospect for prevention with vaccines. Pediatr. Infect. Dis. J. **8**:271–276.
28. **Waltman, W. D., L. S. McDaniel, B. M. Gray, and D. E. Briles.** 1990. Variation in the molecular weight of PspA (pneumococcal surface protein A) among Streptococcus pneumoniae. Microb. Pathog. **8**:61–69.
29. **Wessels, M. R. (Boston, Mass.).** Personal communication.
30. **Wilkinson, H. W., and R. G. Eagon.** 1971. Type-specific antigens of group B type Ic streptococci. Infect. Immun. **4**:596–604.