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Molecular Analysis of Group B Protective Surface Protein, a New Cell Surface Protective Antigen of Group B Streptococci

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Received 23 August 2001/Returned for modification 30 October 2001/Accepted 12 November 2001

Group B streptococci (GBS) express various surface antigens designated c, R, and X antigens. A new R-like surface protein from Streptococcus agalactiae strain Compton R has been identified by using a polyclonal antiserum raised against the R protein fraction of this strain to screen a lambda Zap library. DNA sequence analysis of positive clones allowed the prediction of the primary structure of a 105-kDa protein designated BPS protein (group B protective surface protein) that exhibited typical features of streptococcal surface proteins such as a signal sequence and a membrane anchor region but did not show significant similarity with other known sequences. Immunogold electron microscopy using a BPS-specific antiserum confirmed the surface location of BPS protein on S. agalactiae strain Compton R. Anti-BPS antibodies did not cross-react with R1 and R4 proteins expressed by two variant type III GBS strains but reacted with the parental streptococcal strain in Western blot and immunoprecipitation analyses. Separate R3 and BPS immunoprecipitation bands were observed when a cell extract of strain Compton R was tested with an antiserum against Compton R previously cross-absorbed to remove R4 antibodies. Immunization of mice with recombinant BPS protein by the subcutaneous route produced an efficient antigen-specific response, and immunized animals survived challenge with a lethal dose of a virulent strain. Therefore, BPS protein represents a new R-like protective antigen of GBS.

Group B streptococci (GBS) have emerged as an important cause of infection in neonates that is characterized by a high mortality rate, even in developed countries (1). In the United States alone, more than 15,000 cases and 1,300 deaths due to GBS occur each year (46). The problem of GBS infection of neonates lies in the fast and dramatic course of infection, which can only be treated inadequately with antibiotics (13). Since many women of child-bearing age have vaginal GBS colonization, they are tested during pregnancy for carriage of GBS (31). Besides affecting neonates, GBS cause a number of maternal peripartum diseases and are responsible for serious illness in nonpregnant adults (6). Necrotizing fasciitis and toxic shock-like syndrome due to GBS have recently been reported in adults (12).

GBS strains comprise nine serotypes based on the presence of specific capsular polysaccharides. Of these, serotypes Ia, Ib, II, III, and V have been most prevalent. Approximately 40% of isolates in cases with invasive GBS disease express Ia polysaccharide, and 27% express type III polysaccharide (24). In certain geographic areas, serotype V is emerging as the predominant serotype (14, 19, 30). The occurrence of type V isolates, however, increases with age, whereas that of other serotypes decreases (14, 19, 30). Because of increasing antibiotic resistance and the restriction of antibiotic therapy during pregnancy (33), it is desirable to develop alternatives to antibiotic therapy. Clinical studies have shown that newborns whose mothers have high titers of anti-GBS antibodies are seldom infected (2). An attractive alternative to classic antibiotic therapy could be vaccination of women of child-bearing age to protect newborns against GBS infection (3). Since the GBS capsule plays an important role in virulence, attempts have been made to develop a vaccine based on capsular polysaccharides. However, these vaccination studies have been unsuccessful because of antigenic variation and the low immunogenicity of capsular polysaccharides. A potential solution to this problem can be the use of glycoconjugates as candidate vaccines (43).

Because of the suboptimal immunogenicity of capsule-based vaccines, interest has shifted toward the surface protein antigens of GBS as vaccine candidates or carrier proteins for specific GBS polysaccharides. These antigens include the α and β antigen of the c protein complex (17, 26), an α-like protein (20), the R proteins (10), and protein Rib (38). R proteins are cell surface proteins of GBS that are resistant to certain proteases (10). Four distinct species of R protein in GBS have been described; of these, R4 is the predominant species (10, 44). Most of the isolates of serotype III express R proteins on the surface (10). In animal models, c protein antigens are protective (40). The c protein antigens are expressed by 90% of isolates of types Ia and Ib and by 50% of type II invasive isolates; however, they are rarely expressed by type III GBS (8). Rib protein also confers protective immunity in mice (38). Other R proteins are also biologically important, and a correlation between low levels of maternal antibodies to R proteins and neonatal septicemia has been reported (25). An-
other surface protein from type V GBS which shares N-termini
cell surface R protein electroeluted onto Immobilon P membranes (Millipore) was performed
sequencer (Applied Biosystems). For complete sequencing of both strands of the DNA
other surface protein from type V GBS which shares N-termini

**MATERIALS AND METHODS**

**Bacterial strains, phages, plasmids, and media.** GBS strains were from the culture collection of the University of Minnesota (UM), Minneapolis. R protein

**Antisera.** Rabbit antisera included a polyvalent serum recognizing R3, R4, and

**Immunoprecipitation reactions in agarose.** R proteins from GBS strains were detected in agarose slides by Ouchterlony double-diffusion (DD) immunoprecipitation using Lancefield hot HCl or 0.1% trypsin cell extracts (11). To examine the susceptibilities of the various R proteins to trypsin or pepsin digestion, HCl or 0.1% trypsin cell extracts were treated (1 h at 37°C; pH 8.2) either with 0.2% trypsin or with 0.5 or 0.2% pepsin (pH 2.0 and 4.0, respectively) (11, 38, 44, 45). Enzyme-treated or control (buffer only) samples were then tested to deter-

**DNA manipulations.** Chromosomal DNA from *S. agalactiae* Compton R was

**Immunization and protection studies.** Four-week-old female BALB/c (H-2b)

** SDS-PAGE and Western blot analysis.** Sodium dodecyl sulfate-polyacrylamide
gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (22), and the gel was then stained with Coomassie brilliant blue R250 (Sigma).

**Immunoelectron microscopy.** For preembedding labeling of the BPS protein,
bacteria were grown in Todd-Hewitt broth overnight, centrifuged, and resus-

**Ribosome mapping.** The polyvalent antiserum against R3, R4, and BPS was used

cell surface R protein electroeluted onto Immobilon P membranes (Millipore) was performed

**Postembedding labeling, bacteria were fixed with 0.2% glutaraldehyde and 0.5%

**Isolation and purification of R protein.** BPS (group B protective surface protein) that is expressed by nontypeable/R3, R4, BPS) (Compton 25/60, wild-type Strain 7, and the prototype strain Strain 804, was used as the antigen in immunological studies. For postembedding labeling of the BPS protein, the gene was cloned into pQE30 (Qiagen), and the His-tagged BPS fusion protein, the gene was cloned into pQE30 (Qiagen), and the His-tagged BPS fusion protein, the gene was cloned into pQE30 (Qiagen), and the His-tagged BPS fusion protein, the gene was cloned into pQE30 (Qiagen)
tinylated μ-chain-specific goat anti-mouse IgM and γ-chain-specific goat anti-
mouse IgG (Sigma) and incubated for a further 2 h at 37°C. After four washes, 100 μl of peroxidase-conjugated streptavidin (PharMingen) was added to each well and plates were incubated at room temperature for 1 h. After four washes, reactions were developed using ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sul-
fo nic acid)] in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H2O2.

Nucleotide sequence accession number. The sequence of the gene encoding the BPS protein of GBS has been assigned EMBL accession number AJ133114.

RESULTS

Purification of cell surface R proteins from S. agalactiae Compton R. The purified product obtained after alkaline extraction and fast protein liquid chromatography anion-exchange chromatography gave four bands of 125, 120, 115, and 110 kDa in a silver-stained SDS-PAGE gel. Western blot analysis indicated that all bands were immunoreactive against polyclonal reference R antiserum, with the 125- and 120-kDa bands reacting most prominently. Since the antiserum used was generated against all surface R proteins, it is likely that the less prominent 115- and 110-kDa bands represented other R pro-
teins; this, however, could not be determined, because the antiserum could not differentiate between R3 and R4. Alkaline extraction in the presence of protease inhibitors or further purification by gel filtration did not change the gel pattern. These purified proteins were used to raise a polyclonal anti-
serum for screening of the gene library.

Cloning and nucleotide sequence analysis of the gene encoding BPS protein. A lambda Zap gene library of S. agalactiae Compton R chromosomal DNA was screened using a polyclonal antiserum raised against purified cell surface R proteins isolated from the homologous strain. One clone, which ex-
pressed a protein with an apparent molecular size of 125 kDa that was reactive against the antiserum, was designated pSE3. The 5.0-kb DNA insert of the plasmid was subjected to DNA sequence analysis, which revealed a single open reading frame of 2,937 bp encoding a predicted 105-kDa protein (Fig. 1). A putative Shine-Dalgarno sequence (5′-GAGGAAG-3′) was detected 5 bp upstream of the ATG start codon. Both −35 (5′-TTGGAT-3′) and −10 (5′-TATAAT-3′) consensus sequences were present at least typical for E. coli were detected 94 and 66 bp upstream of the open reading frame, respectively. A putative 39-amino-acid signal sequence was detected at the amino termi-
num of the putative protein and shared DNA similarity with other gram-positive cell surface protein signal sequences (41). The carboxy terminus of the protein contained an LPXTG consensus motif typical of membrane-anchored surface pro-
teins of many streptococci and other gram-positive bacteria (9, 15). Within the carboxy-terminal half of the protein, two identical repeats of 76 amino acids each, separated by a 101-amino-
acid spacer region, were found. After a search of the EMBL database, the protein was found to share limited DNA simi-
ilarity only with the protein of unknown function encoded by the Streptococcus suis mrp gene (36). The R protein analyzed in this study, which was present in cell surface extracts of S. agalactiae Compton R, was immunologically distinct from R1, R3, and R4 (see below) and was thus designated BPS.

Expression and purification of GST-tagged BPS. By use of the 5′- BamHI and 3′-SmaI primers, the bps gene was PCR amplified, excluding coding regions for the amino-terminal signal sequence and carboxy-terminal membrane anchor region, and ligated into the relevant enzyme restriction sites of the pGEX-2T expression vector (Pharmacia) to form pSE4. Induction of E. coli XL1-Blue MRF+ (pSE4) resulted in the expression of GST-tagged BPS with an apparent molecular size of 158 kDa, which was subsequently purified using gluta-
thoniane-agarose affinity chromatography (Fig. 2A). The purified recombinant GST-BPS fusion protein was used to raise a polyclonal rabbit antiserum which recognized the degraded BPS protein with an apparent molecular size of 125 kDa in S. agalactiae Compton R whole-cell extracts, the GST with an apparent molecular size of 32 kDa in E. coli XL1-Blue MRF+ (pGEX-2T) whole-cell extracts, and the 158-kDa degraded recombinant BPS fusion protein in E. coli XL1-Blue MRF+ (pSE4) whole-cell extracts (Fig. 2B, lanes 1 to 3, respectively). A commercially acquired anti-GST monoclonal antibody re-
acted against GST and GST-tagged BPS, but not against native BPS, in S. agalactiae Compton R whole-cell extracts (data not shown).

Western blot analysis using anti-BPS serum. Western blot analysis using BPS antiserum revealed that BPS antigen produced by S. agalactiae Compton R, partially purified Compton R surface proteins, and purified recombinant GST-tagged BPS are degraded following sample preparation, resulting in detection of multiple bands (Fig. 2C, lanes 1, 2, 4, and 6). However, the lack of reactivity of the BPS antiserum against E. coli XL1-Blue MRF+ confirms the lack of cross-reactive antibodies and the purity of the GST-tagged BPS preparation used to prepare the polyclonal antiserum (Fig. 2C, lane 1).

The BPS antiserum was also used to detect BPS in a number of different GBS serotypes. The lack of reactivity of the anti-
serum with S. agalactiae 71-735 (serotype III/R1; Fig. 2C, lane 3) and 76-043 (serotype III/R4; lane 5) revealed that BPS was not immunologically related to R1 and R4. S. agalactiae H4A-0126 (serotype Ia/R1), on the other hand, did express a protein of higher apparent molecular weight which was reactive with the BPS antiserum and produced a degradation profile similar to that of Compton R, suggesting that H4A-0126 also ex-
pressed BPS, that the BPS antigen displayed size variation, and that BPS was expressed in more than one serotype of S. aga-
lactiae (Fig. 2C, lane 4). Further proof of the immunological distinction between R1, R3, R4, and BPS was the fact that the antiserum to R3 and BPS from Compton R did not react with proteins from strain 71-735 or 76-043 (Fig. 2C, lanes 3 and 5).

Cell surface expression of BPS in S. agalactiae Compton R. To confirm and characterize the location of BPS protein on the surfaces of S. agalactiae cells, immunochemistry was carried out by using a monoclonal polyclonal anti-BPS anti-
serum. Immunogold particles were found to be evenly distrib-
uted on the cell surface of the parental S. agalactiae strain Compton R by pre- as well as postembedding techniques (Fig. 3A, C, and D). This indicates that BPS protein is homoge-
neously expressed on the cellular surface. No labeling was found in bacterial cytoplasm. A GBS isolate(76-043/III/R4) that does not express the BPS protein exhibited only a very few gold particles bound to the surface (Fig. 3B). Anti-GST anti-
odies as well as preimmune serum were used as controls and did not reveal any labeling (data not shown).

Ouchterlony DD analysis of S. agalactiae Compton R pro-
FIG. 1. Nucleotide sequence and deduced amino acid sequence of the gene encoding the BPS protein of GBS (EMBL accession number: AJ133114). Putative NC10 and NC35 sequences are boxed, and the Shine-Dalgarno region is underlined. Within the BPS coding region, the initial codons of the two repeats are boxed and the regions coding for the LPXTG membrane anchor consensus sequence and the charged C-terminal tail are underlined. Amino acids are given in one-letter code.
with the purified recombinant protein and the HCl extract of strain Compton R, while the extracts of strains 71-735 (III/R1) and 76-043 (III/R4) did not react with this antiserum (data not shown).

Enzyme susceptibility studies of the BPS from *S. agalactiae* Compton R. To compare the enzyme susceptibility of BPS to that of R3, the HCl extract of Compton R was treated with trypsin or pepsin and then compared by DD to buffer-treated controls. Figure 4B shows the R3 (inside, denser) and BPS (outer, lighter) precipitin bands of the untreated extract (well 1) when the antiserum to R3 and BPS was placed in the center well. Treatment with 0.02% trypsin did not affect the BPS and R3 reactions. Treatment of the extract with 0.2% trypsin (well 5), however, eliminated BPS but did not affect the R3 reaction, while 5% trypsin greatly diminished the R3 reaction (well 4). By contrast, only the BPS precipitin band remained after treatment with 0.2% pepsin at pH 4 (well 3), and neither the R3 nor the BPS reaction was seen after treatment with 0.5% pepsin at pH 2, the optimum and conventional pH for pepsin activity (data not shown). Buffer only at pH 8, pH 4, or pH 2 (data not shown) did not affect either the R3 or the BPS precipitin band.

Vaccination with BPS protein protects mice against challenge with virulent strains of GBS. For immunization studies, His-tagged BPS was used to avoid the immune-modulatory effects of GST. The gene encoding the whole BPS protein was cloned into the pQE30 expression vector, and His-tagged protein was purified by affinity chromatography. Immunization of mice with recombinant BPS protein by the subcutaneous route triggered the elicitation of a very efficient antigen-specific response (Fig. 5A). When the immunized animals were challenged with strain Compton R, approximately 88% of the vaccinated animals survived (Fig. 5B), whereas mice in the control group were not protected (78% lethality).

**DISCUSSION**

In spite of advances in diagnosis and treatment, GBS infections remain a major cause of neonatal mortality and morbidity. Development of an effective vaccine to prevent GBS disease through maternal immunization seems to be a promising strategy for the control of GBS infections. A prerequisite for the development of an effective vaccine is the identification and characterization of potential cell surface targets for therapeutic intervention. Because of the suboptimal potential of capsular polysaccharides, interest has shifted to protein antigens as components of vaccine candidates. A number of surface proteins such as β antigen (17, 18), α protein (27), protein Rib (38), and R proteins (10, 11) have been described. Most of these protein antigens have been shown to be protective either by contributing toward resistance to opsonization (32) or by eliciting protective immunity (21, 27). Elicitation of protection against encapsulated GBS strains (23) underlines the importance of GBS surface proteins as vaccine candidates. In this report we describe yet another GBS R-like surface protein that was cloned from strain Compton R; sequence analysis showed that it possesses all the typical features of gram-positive surface proteins such as the signal sequence (41) and the membrane anchor, which contains the sequence LPXTG (9). The surface location was confirmed by immunoelectron microscopy. Sequence analysis showed no similarity on a protein level with
any known GBS proteins, indicating that this is a novel surface protein; therefore, it was designated BPS. The protein consists of 979 amino acids and contains two identical repeats of 76 amino acids separated by a 101-amino-acid spacer in the C-terminal region. BPS, therefore, belongs to a family of GBS surface proteins with repetitive structures (42). Two other members of this family, namely, protein Rib (38) and α protein (28), are also trypsin resistant and give a ladder-like pattern similar to the BPS pattern in SDS-PAGE and Western blot analysis. BPS, however, showed no sequence similarity to protein Rib or α protein. On the protein level, BPS shows some similarity (26% identity) to the mrp gene from S. suis (36). The function of the mrp gene product is not yet known.

To determine the prevalence of BPS, the protein was expressed as a fusion, purified, and used to raise a polyclonal antiserum. Immunoprecipitation in agarose was useful to determine that BPS was indeed an integral surface antigen of S. agalactiae Compton R and to identify it as a unique protein, separate from R3 and R4 proteins. In early (44) and subsequent (10) work on the R proteins of GBS, strain Compton R was identified as having only R3 and R4 proteins. When we tested our HCl extracts with Prague anti-Compton R serum, which was used in those studies, the BPS precipitin reaction was weak and practically fused with that of R3, making it difficult to recognize them as two distinct lines. However, separation and identification of three separate proteins in the HCl and trypsin extracts of Compton R were possible with our new polyclonal rabbit antiserum. Comparison of the reactions obtained with our new antiserum with those obtained with the antiserum from Prague showed that, although they were somewhat obscured, the Prague antiserum also contained antibodies to BPS (data not shown). This further confirmed that BPS is a new protein that is separate from R3 and R4, the previously recognized proteins of Compton R.
The results from the enzyme digestion studies provided additional support for the fact that BPS is a separate protein of Compton R. DD results indicated that mild trypsin treatment affected BPS more than R3. In contrast, the results from mild pepsin treatment of BPS at suboptimal conditions (pH 4) indicated that R3 was more pepsin susceptible than BPS. This reduced susceptibility of BPS to pepsin digestion is similar to that of R4 protein (11) and protein Rfb, since treatment with pepsin at suboptimal pH conditions was used to characterize the latter and to compare it to the α component of the c protein (38), another trypsin-resistant protein of GBS (7).

Finding BPS in GBS strains such as H4A-0126 and H4A-0148 demonstrates that this protein of Compton R is found in wild GBS strains of other serotypes and protein profiles. Our results from examination of 1,627 human GBS isolates by double immunodiffusion and Southern hybridization indicated that BPS was found in 7.4% of serotype Ia/R1 isolates. 6.8% of serotype II isolates, and 5% of serotype V isolates (A. E. Flores, S. Erdogan, G. S. Chhatwal, C. J. Baker, S. Hillier, and P. Ferrieri, Abstr. XIVth Lancefield Int. Symp. Streptococci Streptococcal Dis., abstr. O2.8, 1999). The presence of both R1 and BPS in the serotype Ia isolates was analogous to the presence of both R1 and R4 in the majority of serotype V isolates (8). Furthermore, these results indicated that BPS was a marker found in recently isolated colonizing human isolates and that, as such, it may be useful in their characterization (7).

The results obtained demonstrated that the BPS protein is an antigen able to confer protective immunity against GBS. In order to evaluate whether the BPS protein can also confer protection against challenge with a heterologous strain, immunized animals were challenged with strain B176 (Ia, BPS). While 67% of the vaccinated animals survived, only 22% of the
mice in the control group survived (data not shown). These results confirmed that immunization with the BPS protein can also protect against heterologous challenge (P ≤ 0.05).

Considering that the natural portal of entry for GBS is the mucosa from the respiratory and genitourinary tracts, it seems particularly attractive to stimulate not only an efficient systemic but also a local mucosal response following vaccination. This may lead to a more efficient protection of newborns by two different mechanisms, namely, (i) passive transfer of maternal antibodies leading to protection against disease and (ii) reduction of the risk of maternal colonization (i.e., infection) by the presence of vaginal antibodies. Of importance is the fact that BPS elicits a strong IgG response; this isotype can cross the placenta and therefore may provide passive protection. It has also been demonstrated that due to the existence of a mucosal network, coadministration of vaccine antigens and mucosal adjuvants by the intranasal route can lead to the elicitation of local responses in the urogenital tract (16). Preliminary studies from our group confirmed that protective immunity can also be achieved following vaccination by the intranasal route with the BPS protein in combination with the cholera toxin B subunit as a mucosal adjuvant.

Different serotypes of GBS express different surface proteins, which can become targets for the elicitation of protective immune response. An effective vaccine, therefore, should contain more than a single antigen in order to confer protection against predominant circulating serotypes. Thus, BPS protein, which is expressed by isolates of serotypes Ia, II, and V, may represent an attractive candidate antigen for incorporation in anti-GBS vaccine formulations.

ACKNOWLEDGMENTS

This work was supported in part by research contract N01 AI25152 from the National Institutes of Health (to P.F. and A.E.F.). S.E. gratefully acknowledges a Ph.D. fellowship from the Boehringer-Ingelheim Fond. M.J.W. and P.K.F. thank the Alexander von Humboldt Foundation for fellowships. A travel grant from the University of Melbourne to M.J.W. is gratefully acknowledged.

We thank E. Moore for help in DNA sequencing and A. Müller for help in animal experiments. We acknowledge receiving two BPS strains from C. J. Baker, Baylor College of Medicine.

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Editor: D. L. Burns