

2002

Characterization of host-bacteria interactions contributing to group B streptococcus colonization

Jennifer Marie Smith

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**CHARACTERIZATION OF HOST-BACTERIA
INTERACTIONS CONTRIBUTING TO GROUP B
STREPTOCOCCUS COLONIZATION**

DISSERTATION

Submitted to the Graduate College

of

Marshall University

In Partial Fulfillment of the Requirements for

The Degree of Doctor of Philosophy

By

Jennifer Marie Smith

Huntington

West Virginia

2002

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ACKNOWLEDGEMENTS

My deepest gratitude goes to my mentor and friend Dr. Susan Jackman for her unending patience, enthusiasm, and guidance. I also thank the other members of my committee for their support as well as: Dr. Bryan Larsen for sparking my interest in infectious disease research and his continued guidance despite the physical distance separating us; Dr. Elizabeth Bryda for her molecular biology expertise and the use of numerous pieces of equipment; Dr. Beverly Delidow for her assistance in designing and analyzing the IL-6 binding assay; Dr. SueEllen Ferraris for her assistance in flow cytometry data analysis; and Dr. Darshana Shah for her insight in working with human neutrophils.

In addition to my committee members I owe thanks to several members of the Departments of Microbiology, Immunology, & Molecular Genetics and Obstetrics and Gynecology. I thank Dr. Robert Nerhood and Dr. David Chaffin for providing access to patients in the obstetric clinics and for financial support. I thank Dr. Don Primerano and Dr. Hongwei Yu for their assistance in DNA sequence analysis. I thank Dr. Pamela Staton and Sarah Price for their technical expertise, encouragement, and friendship. I thank Sandra White for her assistance in acquiring patient samples and for her support.

I also thank my family and friends for their unfailing belief in my abilities. Without their faith and love none of my accomplishments would have been possible.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CAMP	Christie, Atkins, Munch-Petersen
cDNA	complementary DNA
CFU	colony forming units
CR1	complement receptor 1
CR3	complement receptor 3
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
GBS	group B streptococcus
GBS+	group B streptococcus-colonized
GBS-	group B streptococcus-noncolonized
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
kb	kilobases
kDa	kilodaltons
MBL	mannose binding lectin
mRNA	messenger RNA
μg	microgram

μl	microliter
ml	milliliter
M-MLV	Moloney Murine Leukemia Virus
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ng	nanogram
NO	nitric oxide
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with Tween 20
PCR	polymerase chain reaction
PMA	phorbol myristate acetate
RAP-PCR	random arbitrarily primed polymerase chain reaction
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
SSO	sequence-specific oligonucleotide
TBS-T	tris-buffered saline with Tween 20
THB	Todd-Hewitt broth
TLR	Toll-like receptor
TMAC	tetramethylammonium chloride
TNF-α	tumor necrosis factor-α

ABSTRACT

Group B streptococcus (GBS) is the leading cause of life-threatening bacterial infections during the first three months of life. GBS is also a frequent cause of maternal postpartum infections. Both types of infections stem from maternal vaginal and/or rectal colonization with GBS in the perinatal period. Limited information is available concerning how the colonization process occurs and what role the host immune system may play in the establishment of persistent colonization by GBS. The complex interactions between the immune system and GBS normally should end in clearance of the bacteria. However, since colonization by GBS occurs in a large number of women it can be surmised that the immune defenses are not functioning in a manner consistent with a protective response or that GBS is in some fashion undermining them. Investigations examining interactions between GBS and components of the innate immune system support this hypothesis. Measurement of phagocytosis and respiratory burst capacities revealed that monocytes, in particular, from these women internalize larger quantities of bacteria while the antimicrobial superoxide is shunted to an extracellular location. This data indicated that phagocytes from colonized women might become a protective niche for GBS upon engulfment. Additional data also showed that these same women (GBS-colonized) had significantly increased levels of mannose binding lectin (MBL) in their sera and that high levels of MBL significantly enhanced phagocytic engulfment of GBS. While these data support the idea that the host immune system is not functioning as it normally should to prevent infection and colonization, other data provided evidence that GBS is capable of using components of the immune response to its own benefit. GBS bound interleukin-6 (IL-6) and used the biologically active pro-inflammatory cytokine to

enhance its growth and the expression of at least one virulence factor, the C5a-ase. GroEL, a protein implicated in pathogenesis of a number of other bacterial species, was also increased by IL-6 exposure to GBS. Understanding how the immune system and GBS interacts is key to elucidating the mechanisms that underlie the colonization and infection processes as well as may aid in the identification of targets for potential treatment and prevention strategies.

CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Group B streptococcus was originally identified as a cause of bovine mastitis in the 1800s. The first documented case of human group B streptococcus (GBS) infection was recorded in 1938 (Weisman et al, 1992). By the 1970s, GBS was recognized as a major cause of life-threatening neonatal bacterial infections.

During this period GBS research has focused primarily on prevention and treatment strategies for neonatal GBS disease. While some attention has been paid to phagocytosis of GBS, epithelial cell invasion by the bacteria, and specific virulence factors, many areas of immunity to GBS remain relatively underexplored. More detailed knowledge of how the immune system and GBS interact with one another will aid in the development of models of GBS pathogenesis as well as identify potential targets for future therapeutic and preventative measures.

The aim of this study was to examine the interactions between group B streptococcus and components of the innate immune system including engulfment and respiratory burst activity by phagocytes, mannose binding lectin gene polymorphisms and opsonic capacity, and the effects of pro-inflammatory cytokines effects on GBS growth and virulence. These investigations were designed to test the hypothesis that interactions between GBS and the innate immune system may contribute to the GBS colonization process, or at least does respond in a way that would prevent colonization.

LITERATURE REVIEW

GBS Microbiology

Streptococcus agalactiae, more commonly referred to as group B streptococcus (GBS), is a gram-positive, facultative anaerobic bacterium of the family *Streptococcaceae*. GBS can be found as a component of the normal flora of the adult gastrointestinal tract, urogenital tract, and upper respiratory tract.

Phenotypically GBS colonies appear flat, grayish-white and glistening on solid media. Gram stain smears of GBS isolates reveal chains or pairs of cocci.

Identification of GBS from individuals suspected of GBS infection is accomplished by culture of the bacterium from normally sterile body sites (blood, urine, cerebrospinal fluid). Presumptive identification of GBS is based on colony morphology, presence of β -hemolysis, hippurate hydrolysis, and a positive CAMP test. Confirmation of GBS identification relies on detection of the group B antigen by latex agglutination.

GBS may be categorized into serotypes based on the capsule polysaccharide composition. To date there have been at least 9 different GBS serotypes (Ia, Ib, II, III, IV, V, VI, VII, and VIII) identified. Serotypes Ia, Ib, II, III, and V are most commonly isolated and most often associated with GBS disease, although all of the serotypes are capable of causing infection (Harrison et al, 1998; Andrews et al, 2000; Tyrell et al, 2000; Davies et al, 2001).

Risk Factors

In 1996-7 the Centers for Disease Control, the American College of Obstetricians and Gynecologists and the American Academy of Pediatrics released guidelines for identifying pregnant women at risk for having an infant with GBS disease (Schuchat et

al, 1996; ACOG, 1996; AAP, 1997). Delivery of an infant with GBS disease in a previous pregnancy is one of the six criteria. Premature rupture of the amniotic membrane, prolonged rupture of the amniotic membrane, intrapartum fever, GBS bacteruria, and GBS colonization late in pregnancy are the other risk factors.

In adults, risk factors for GBS infection differ from those for neonatal disease. In pregnant women, prolonged rupture of the amniotic membranes, intrauterine monitoring, and multiple vaginal exams during labor are risk factors for peripartum infections (Schuchat et al, 1998). Having an underlying condition like diabetes mellitus, cardiac disease, or cancer; immunosuppression due to HIV infection or immunosuppressive therapy; or being elderly increase the risk of acquiring GBS disease (Michel, 1990).

Race also appears to be a risk factor for GBS disease, as there is a higher colonization rate in African American women and a higher neonatal infection rate in African American infants (Schuchat et al, 1998). This association between African American race and GBS disease may reflect the increased incidence of low birth weight and premature infants in this population.

Maternal Screening

Because prophylactic antibiotic treatment given during labor to women identified as “at risk” has been shown to dramatically decrease transmission to the neonate (Boyer et al, 1986; Gilson et al, 2000), many obstetricians perform GBS screening cultures at 35-37 weeks during pregnancy. Typically, swabs are taken from the vagina of the patient. However, cultures taken from both the vagina and rectum increase the accuracy in identifying women that carry GBS (Quinlan et al, 2000). Most often swabs are taken by the obstetrician or nurse, but studies by Mercer et al (1995) show that specimens

collected by the women themselves are equally accurate for determining GBS colonization as those collected by the clinical staff.

In the clinical microbiology laboratory, the anogenital swabs are inoculated into a selective medium. Routinely Lim broth or Todd-Hewitt Broth supplemented with gentamicin and nalidixic acid are used to inhibit growth of other normal flora (Dunne et al, 1998). The selective media culture is then streaked onto sheep's blood agar. Presumptive identification of GBS is based on colony morphology and presence of β -hemolysis. GBS confirmation involves agglutination with anti-GBS antibody-coated latex beads (Michel, 1990).

GBS Disease

In Neonates

In infants, GBS disease is categorized based on the time of the appearance of symptoms. Early-onset neonatal disease, which accounts for 70-80% of all neonatal GBS disease, occurs in the first seven days following birth (Schrage et al, 2000; Spellerberg, 2000). Incidence of early-onset disease in the United States was 0.5-2 per 1000 live births with a fatality rate of 4-5% in the 1990s (Schrage et al, 2000). Transmission of GBS resulting in early-onset disease can occur by aspiration of contaminated amniotic fluid during parturition or *in utero* by GBS that have crossed the intact amniotic membranes. The primary syndromes seen in early-onset neonatal GBS disease are bacteremia, pneumonia, and meningitis (Ferrieri, 1985; Schrage et al, 2000).

Meningitis and bacteremia due to GBS infection in an infant between 7 days and 3 months of age is considered late-onset neonatal GBS disease. Incidence of late-onset disease in the United States was 0.3-0.5 per 1000 live births with a fatality rate of

approximately 3% in the 1990s (Schrag et al, 2000). The mode of transmission of late-onset neonatal GBS disease is unclear, but is believed to be by a community or nosocomial route.

Pneumonia associated with GBS disease presents with signs of respiratory distress within the first 24-48 hours following delivery. Infants with GBS pneumonia have tachypnea, cyanosis, apnea, acidosis, and leukopenia (Ferrieri, 1985). Because of the similarity of symptoms, GBS pneumonia is often misdiagnosed as hyaline membrane disease (Michel, 1990).

Although meningitis is seen with early-onset GBS disease, the majority of cases are associated with late-onset disease. Signs of GBS meningitis include lethargy, fever, vomiting, and seizures (Michel, 1990). GBS enter the central nervous system by invading brain microvascular endothelial cells (Nizet et al, 1997). This invasion leads to cellular injury including necrosis in the cerebral cortex and apoptosis in the dentate gyrus of the hippocampus (Kim et al, 1995; Leib et al, 1996). This damage, due to the inflammatory response induced by GBS, can lead to permanent neurologic deficits in infants surviving GBS meningitis (Michel, 1990).

Septic shock results from an overwhelming inflammatory response to GBS bacteremia. GBS sepsis is characterized by cardiovascular collapse, diffuse intravascular coagulation, and multiorgan failure (Michel, 1990; Spellerberg, 2000). Many of these alterations are mediated by proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α), or by other inflammatory molecules induced by these cytokines (Vallejo et al, 1996; von Hunolstein et al, 1997; Kwak et al, 2000; Berner et al, 2001).

In Pregnant Women

In the 1990s, GBS infections in pregnant women accounted for 6-15% of all invasive GBS disease (Schuchat et al, 1998; Schrag, 2000). The majority of these infections were bacteremia, meningitis, osteomyelitis, and endocarditis (Schuchat et al, 1998). GBS also causes urinary tract infections, postsurgical infections, amnionitis, and endometritis in pregnant women (Schuchat et al, 1998). Late term spontaneous abortion and stillbirth have been linked with GBS in the genital tracts of pregnant women (Daugaard et al, 1988).

In Nonpregnant Adults

The mean age of adults with GBS disease is approximately 60 years and the fatality rate is the highest for persons over 65 years of age. Interestingly, underlying conditions like diabetes and cancer only increase the risk of GBS disease in the over fifty population by 4-6 fold (Schuchat et al, 1998). However, in individuals 20-64 years old diabetes increases risk by 11-30 fold and cancer increases risk by 25 fold in individuals 20-49 years old (Schuchat et al, 1998). The majority of GBS disease in nonpregnant adults is pneumonia, bacteremia, and soft tissue infections (cellulitis, osteomyelitis, septic arthritis, wound infections) (Schuchat et al, 1998).

Treatment

Current treatment for GBS disease relies on GBS's continued susceptibility to penicillin G and ampicillin. Routinely, high doses of ampicillin with an aminoglycoside are given intravenously. For patients with a known penicillin allergy, erythromycin, clindamycin, or chloramphenicol have been used as substitutes (Michel, 1990). However, in recent years a significant increase in the number of GBS isolates resistant to

erythromycin and clindamycin has been reported (Morales et al, 1999; Lin et al, 2000; De Azavedo et al, 2001; Murdoch et al, 2001). Mechanical ventilation, seizure control, and blood transfusions may be required in addition to antibiotic therapy (Michel, 1990).

Prevention

Intrapartum antibiotic therapy is used as a measure for prevention of early-onset GBS disease. However, it does not protect against late-onset neonatal or adult GBS disease. Vaccination against GBS is the best solution. The ideal GBS vaccine would elicit both systemic and mucosal responses as well as generate antibodies capable of passing through the placenta to the fetus. Initial attempts at designing a vaccine against the GBS type III capsule polysaccharide did not produce a sufficient response as only 40% of the women vaccinated generated the antibody titers necessary for protection (Baker et al, 1988). More recent attempts using protein carrier conjugates, like tetanus toxoid (Kasper et al, 1996; Wessels et al, 1998; Paoletti et al, 2001) and cholera toxin B subunit (Shen et al, 2000; Shen et al, 2001) show promise, as the vaccines have proven efficacious in the maternal immunization-neonatal challenge mouse model. These studies also demonstrate that the route of administration impacts the type of antibody response. Shen et al (2000) provide evidence that intranasal immunization elicits IgG and IgA responses in the lung and IgG in the serum, oral immunization elicits IgG and IgA in the intestines and IgG in the serum, vaginal immunization elicits IgA in the vagina and IgG in the serum, and rectal immunization elicits IgA in the rectum, vagina, and intestines and IgG in the serum.

Other recent vaccine designs are focusing on creating a multivalent vaccine that includes capsule polysaccharide antigens from the most commonly isolated GBS

serotypes or vaccines that include the known immunogenic GBS surface proteins. Epidemiologic studies of GBS serotype distribution in large medical centers indicate that a vaccine against types Ia, Ib, II, III, and V would protect 80-95% of the population (Harrison et al, 1998; Andrews et al, 2000; Tyrell et al, 2000; Davies et al, 2001). A study in a small community hospital in Huntington, WV, confirms that this type of multivalent GBS vaccine would provide protection against the most common serotypes in this setting as well (Smith et al, in submission, 2002). Because they are expressed on the majority of GBS isolates, inclusion of the α -C and Rib proteins, two surface-expressed proteins thought to function as virulence factors, in a vaccine would alleviate the need for a carrier protein as well as elicit a protective response to additional GBS antigens (Larsson et al, 1996; Gravekamp et al, 1999).

Innate Immunity

The host innate immune response provides the earliest line of defense against colonization and infection as well as provides signals that direct the adaptive response to follow. Innate immunity is mediated by a series of pathogen-nonspecific interactions with serum proteins and phagocytes.

Complement System Activation

Complement, a system of serum proteins produced mainly by the liver, is activated by a cascade of proteolytic cleavages. Products from these cleavages mediate a variety of functions that initiate an inflammatory response and enhance clearance of microbes. C3a, C4a, and C5a promote chemotaxis of phagocytes to the site of infection as well as induce the release of inflammatory mediators by granulocytes. C3b acts as an opsonin to enhance phagocytosis.

The complement system may be activated by three pathways. The classical pathway of activation requires microbe-specific antibody, which interacts with C1q and its associated serine proteases, C1r and C1s. This complex cleaves other components of the complement system leading to the formation of the C3 convertase, which cleaves C3 creating the C3b opsonic product.

The alternative pathway of complement activation results from spontaneous hydrolysis of C3 to C3b that then deposits on the microbial cell surface. This C3b interacts with additional complement proteins to generate a complex with C3 convertase activity, which amplifies the production of C3b and increases the availability of opsonin.

The final pathway of complement activation is the lectin pathway. Lectins bind to carbohydrates on the microbe's surface and initiate a proteolytic cleavage cascade in a manner similar to the classical pathway.

The best-characterized member of the lectin family is mannose binding lectin (MBL). MBL is a Ca^{2+} -dependent lectin composed of oligomers of trimeric protein subunits. Its carboxyl-terminal carbohydrate binding domain recognizes N-acetylglucosamine, mannose, N-acetylmannosamine, fucose, maltose, or glucose on the surface of various bacterial, viral, and fungal organisms (Holmskov et al, 1994). MBL also contains a collagen helix-like domain that is required for oligomerization and complement activation (Kurata et al, 1993; Wallis et al, 1999; Heise et al, 2000). The MBL collagenous domain interacts with the mannose binding lectin-associated serine proteases (MASPs) (Thiel et al, 2000; Thielens et al, 2001). The MBL-MASP complex activates C2 and C4 to form the C3 convertase, which in turn produces C3b to enhance phagocytosis (Gadjeva et al, 2001).

In addition to activating the complement system, MBL itself acts directly as an opsonin. MBL bound to one of its carbohydrate ligands on a microbial cell surface interacts with a phagocyte surface receptor triggering engulfment (Turner, 1996).

Phagocytosis

Phagocytosis is a multi-step process mediated primarily by monocytes and neutrophils. Phagocytosis is initiated when receptors on the phagocyte cell surface recognize their ligands. The ligands for these phagocyte receptors range from microbial cell wall components (polysaccharides, lipoteichoic acid, lipoproteins) to complement proteins to immunoglobulins (Unanue, 2002). Although these receptors each engage different microbial components, several working together may constitute the functional recognition unit (Zhou et al, 1993; Zhou et al, 1994; Petty et al, 1996).

Receptors that recognize patterns common to microorganisms and those that recognize complement-opsonized microorganisms are crucial to the process of phagocytosis. An example of a family of receptors that recognize various components on the microbe's surface is the Toll-like receptors (TLRs). Different members of the TLR family recognize different types of microorganisms. For example, TLR-4 recognizes gram-negative bacteria while TLR-2 recognizes gram-positive bacteria and yeasts (Unanue, 2002). Upon interacting with their specific ligands, TLRs initiate a series of signaling events leading to phagocyte activation.

Complement-opsonized microbes interact with several complement receptors on the phagocyte depending on which complement component has been deposited on the organism's surface. Complement receptor 1 (CR1) ligands include C3b and C4b (Fallman et al, 1993). The primary ligand for complement receptor 3 (CR3) and

complement receptor 4 (CR4) is the inactivated C3 cleavage product designated iC3b (Arnaout, 1990). The receptor that recognizes the C1q component also acts as the receptor for mannose binding lectin (Holmskov et al, 1994). Engagement of these receptors leads to engulfment of the microbe and in some cases to activation of additional pathways that impact the immune response.

Interaction between the microbe and the phagocyte's receptor(s) results in internalization of the microbe in a phagosome. Phagosomal maturation involves fusion with lysosomes and creation of a microbicidal environment, including low pH, lytic enzymes, nutrient deprivation, and reactive metabolites. The introduction of several lytic enzymes and antimicrobial peptides into the phagosome occurs upon fusion with the lysosome. These degradative proteins include proteases, acid hydrolases, lysozyme, and the defensins (Brown et al, 2002).

Nutrient deprivation, particularly limitation of iron, acts as a microbicidal mechanism. Iron limitation occurs not only by keeping the microbe within the phagosome, but also by a protein called natural resistance-associated protein 1 (Nramp1). Nramp1 is relocated from the cytoplasm to the phagosomal membrane after engulfment of the microorganism and functions to remove iron from the phagosome (Brown et al, 2002).

As phagocytosis proceeds, reactive oxygen and nitrogen metabolites are generated in an effort to kill the engulfed microbe. A phagosome membrane-bound NADPH oxidase converts O_2 to superoxide. The superoxide is then converted to other highly bactericidal compounds, including hydroxyl radicals, hydrogen peroxide, and hypochlorous acid (Brown et al, 2002).

The reactive nitrogen species nitric oxide (NO) is produced by inducible nitric oxide synthase from L-arginine. Upon interaction with superoxide another toxic nitrogen metabolite, peroxynitrite, is formed. Reactive nitrogen species have been shown to control a number of infectious diseases in mouse models (Brown et al, 2002). However, induction of sufficiently high levels of NO by human phagocytes has yet to be demonstrated.

The anticipated outcomes of the phagocytic process are destruction of the engulfed microorganism, activation of additional immune effector mechanisms, and antigen presentation leading to an adaptive (antigen-specific) immune response.

A number of diseases are associated with deficiencies of certain components of the phagocytic machinery. Deficiencies in components of the classical pathway predispose individuals to infections with pyogenic bacteria, while C3 deficiencies are associated with severe, recurrent bacterial infections. Deficiencies in mannose binding lectin are associated with a multitude of serious, recurrent infections as well as some autoimmune disorders (Holmskov et al, 1994; Epstein et al, 1996; Turner, 1996). A deficiency in the subunits of CR3 produces phagocytes defective in adhesion, chemotaxis, and phagocytosis leaving afflicted individuals susceptible to potentially fatal bacterial infections (Arnaout, 1990). A deficiency in the NADPH oxidase complex that generates reactive oxygen species results in chronic granulomatous disease and impaired killing of microbes within the phagosome (Malech et al, 1987). These examples underscore the importance of the phagocytic machinery in providing early protection against infection and/or colonization.

Immunity to GBS

Antibody- and/or complement-mediated phagocytosis have been shown to be the most effective immune mechanisms for prevention of GBS infection (Baker et al, 1976; Shigeoka et al, 1978; Shigeoka et al, 1983; Kallman et al, 1998; Campbell et al, 2000). Antibodies to the GBS serotype-specific capsule polysaccharide are highly effective in mediating uptake of GBS as well as inducing respiratory burst activity of phagocytic cells (Shigeoka et al, 1978; Kallman, 1998). Antibodies to other GBS surface antigens, like the C and Rib proteins, elicit a protective response in murine models of GBS infection (Michel et al, 1991; Madoff et al, 1992).

In addition to GBS-specific antibodies, complement is required for elimination of GBS. Shigeoka et al (1978) demonstrate that activation of the classical complement pathway is necessary for phagocytosis of serotypes Ia and II GBS, but not for serotype III GBS. Shigeoka et al (1978) also demonstrate that serotype III GBS fail to activate the alternative complement pathway. The inability of type III GBS to activate the alternative complement cascade is attributed to the high sialic acid content of its capsule (Marques et al, 1992).

In the absence of opsonins, such as GBS-specific antibodies and complement, phagocytic engulfment of GBS may occur via CR3. Uptake of GBS by murine macrophages is inhibited by anti-CR3 monoclonal antibodies (Antal et al, 1992). Cuzzola et al (2000) report that anti-CR3 monoclonal antibodies inhibit secretion of tumor necrosis factor- α , one of the major cytokines produced during GBS infection, in response to GBS. Albany et al (2000) demonstrate that GBS interact with a lectin-like site on CR3 in the absence of opsonization. The GBS ligand for CR3 has yet to be

characterized, but may in fact be the group specific polysaccharide according to Cuzzola et al (2000).

Following internalization in murine macrophages, GBS may survive intracellularly for 24-48 hours (Valentin-Weigand et al, 1996; Cornacchione et al, 1998). This intracellular survival may be due in part to GBS's ability to inhibit the protein kinase C signaling pathway which leads to macrophage activation (Cornacchione et al, 1998).

Phagocytosis not only leads to microbial destruction but also to production of cytokines. Cytokines are pleiotropic signaling molecules produced by many host cell types. The functions of cytokines include initiation of inflammation, activation of effector cells and induction of the adaptive immune response. Although cytokines help control the outcome of the immune response to a microbe, overproduction can actually result in disease. GBS septic shock is caused by an overwhelming production of cytokines with pro-inflammatory properties. Several studies provide evidence that tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) are produced in response to GBS or antigens derived from GBS and that these cytokines are key mediators in the development of neonatal GBS sepsis (De Bont et al, 1993; Williams et al, 1993; Peat et al, 1994; Vallejo et al, 1996; Von Hunolstein et al, 1997; Kwak et al, 2000; Berner et al, 2001). De Bont et al (1993) and Berner et al (2001) also report that infants with GBS sepsis have elevated levels of these pro-inflammatory cytokines. Tissi et al (1999) observe high concentrations of IL-1 β and IL-6 in the joints of mice with group B streptococcal arthritis. Reisenberger et al (1997) report that human amnion cells secrete IL-6 and IL-8 in response to GBS.

GBS Virulence Factors

Colonization and infection by a bacterium is related to its ability to survive within the host environment and avoid the host's immune system. Those bacterial products that function in such a capacity are referred to as virulence factors.

Capsule

The capsule is considered the major GBS virulence factor. GBS isolates can be categorized into serotypes (Ia, Ib, II-VIII) based on their capsule polysaccharides. The GBS capsule consists of glucose, galactose, N-acetylglucosamine, and sialic acid with each serotype differing in monosaccharide ratios and linkages (Rubens et al, 1990). Serotype VIII differs in that N-acetylglucosamine is replaced by rhamnose and serotype VI completely lacks N-acetylglucosamine (Spellerberg, 2000). Serotypes Ia, Ib, and III possess sialic acid as the terminal sugar in the capsule polysaccharide.

A 15.5kb region of the GBS chromosome encodes the 16 genes for the capsule synthesis operon (*cps*). *cpsA*, B, C, and D function in determination of polysaccharide chain length. *cpsE* and F are structural genes for monosaccharide synthesis, while *cpsG*, I, J, and K are structural genes for synthesis of the oligosaccharides from activated monosaccharides. *cpsL*, *neuB*, *neuC*, and *neuA* function in transport and sialic acid biosynthesis. *cpsH* is the capsule polysaccharide polymerase, which confers serotype specificity. Upstream of *cpsA* is *cpsY*, which acts as a transcriptional regulator for the *cps* operon (Chaffin et al, 2000).

All serotypes are capable of causing invasive GBS disease, but 50-60% of all neonatal disease is due to infection with serotype III isolates (Ferrieri, 1990). Because of the association of serotype III with the majority of neonatal GBS infections, type III

isolates have been used primarily to investigate the role of the capsule in GBS pathogenesis.

Serotype III GBS treated with neuraminidase to remove sialic acid are more susceptible to serum antibody opsonization and have decreased virulence (Shigeoka et al, 1983). Additional studies by Rubens et al (1987) and Wessels et al (1989) using asialo and unencapsulated serotype III mutants generated by transposon mutagenesis demonstrate that the loss of capsule sialic acid leads to avirulence in a neonatal rat model of GBS sepsis. These studies emphasize the critical role the capsule, in particular the sialic acid, plays in GBS pathogenesis.

Bacterial capsules protect the microbe from the host's immune system response, particularly phagocytosis. Phagocytic killing of GBS is most efficient when serotype-specific antibody is present (Shigeoka et al, 1978). However, most women do not possess specific antibody titers high enough to be protective (Vogel et al, 1980).

In the absence of serotype-specific antibody, activation of the alternative complement pathway should facilitate clearance of GBS. However, GBS serotype III strains fail to activate the alternative complement pathway (Marques et al, 1992). GBS inhibition of the alternative complement cascade is due to the capsule's sialic acid, which alters the affinity of the C3b fragment for regulatory components and ultimately limits the C3 deposited on the surface (Marques et al, 1992).

Hemolysin/Cytolysin

One of the most common presumptive tests for identification of GBS is β -hemolysis. Despite the fact that the β -hemolysin is produced by approximately 99% of all GBS strains (Rubens et al, 1990), characterization of the molecule has proven

difficult. The hemolysin appears to be unstable when extracted from the cell unless a high molecular weight carrier (albumin, starch, Tween 80) is present (Marchlewicz et al, 1980; Dal et al, 1983). Certain general characteristics including protease sensitivity (Marchlewicz et al, 1980; Dal et al, 1983) and inhibition of hemolytic activity by phospholipids (Tapsall et al, 1991) vary depending on the carrier used in hemolysin preparation.

Due to the difficulties encountered in attempts at biochemical characterization of the GBS hemolysin, genetic identification has been recently undertaken. The GBS β -hemolysin is coded for by a 13 gene operon designated *cyl*. Spellerberg et al (1999) identified eight genes in the *cyl* locus by transposon mutagenesis. *cylA* and *cylB* encode an ABC transporter. *cylD*, *cylG*, and *cylZ* function in fatty acid synthesis while *acpC* encodes an acyl carrier protein. *cylX* and *cylE* functions were undetermined. Pritzlaff et al (2001) identify additional *cyl* genes by selecting *E. coli* DH5 α colonies that gained β -hemolysis activity upon transformation with plasmids containing fragments of the GBS chromosome. *cylF* has homology with an aminomethyltransferase; *cylH* and *cylI* have homology with 3-ketoacyl-ACP synthases; *cylJ* has homology with a glycosyltransferase; and *cylK* has no known homology. In addition to extending Spellerberg's work (1999) by identifying 5 additional genes in the *cyl* operon, Pritzlaff et al (2001) demonstrate that the *cylE* gene, while having no known homologs, is sufficient for a β -hemolytic phenotype. The sequence of *cylE* does not display any motifs similar to known bacterial toxins, indicating that the GBS β -hemolysin may possess a novel mechanism of action.

Electron microscopy indicates that the GBS β -hemolysin acts as a pore-forming toxin. Wild-type and hyper-hemolytic GBS strains cause membrane disruption and cell

swelling in A549 lung epithelial cells while nonhemolytic GBS did not cause injury (Nizet et al, 1996). Hemolytic activity has also been correlated with damage to McCoy cells (Tapsall et al, 1991), human brain microvascular endothelial cells (Nizet et al, 1997), mouse peritoneal macrophages, and human macrophages (Fettucciari et al, 2000). The damage to murine and human macrophages leads to apoptosis. Fettucciari et al (2000) propose that the GBS β -hemolysin creates disruptions in the macrophage membrane, which leads to a Ca^{2+} influx and the induction of a caspase-independent pathway of apoptosis. This may represent another GBS mechanism for circumventing the host immune response.

C5a-ase

Neonatal GBS infections often show little infiltration of neutrophils at the site of infection (Hemming et al, 1976). Hill and colleagues (1988) describe inactivation of C5a by GBS and hypothesized that the lack of neutrophil accumulation in the inflammatory foci in GBS infections is due to a C5a-ase enzyme similar to that of *Streptococcus pyogenes*. GBS inactivation of the chemoattractant C5a occurs by proteolytic cleavage of C5a between histidine 67 and lysine 68 thereby disrupting the neutrophil receptor binding site (Bohnsack et al, 1991). The C5a-ase gene, designated *scpB*, was cloned and characterized by Chmouryguima and colleagues in 1996. This 126-kilodalton serine proteinase inactivates C5a from humans, monkeys, and cows, but not C5a from rodents, sheep, or pigs (Bohnsack et al, 1997). Bohnsack et al (1997) also demonstrate that C5-deficient mice reconstituted with human C5 and infected with C5a-ase-positive GBS had decreased neutrophil infiltration compared to those infected with C5a-ase-negative GBS. This study implies that the GBS C5a-ase is in part responsible for the diminished immune

response to GBS in infants because of the reduced numbers of cells at the site of infection that are available for clearance of GBS.

Hyaluronate lyase

Since many neonates with life-threatening GBS infections present with symptoms within hours after birth, it is likely that infection occurred *in utero*. The main barrier to *in utero* infection is the amniotic membrane, which is rich in hyaluronic acid. Hyaluronic acid is also a major component of connective tissue. Hyaluronate lyases are considered spreading factors because of their ability to cleave hyaluronic acid between N-acetyl- β -D glucosamine and D-glucuronic acid residues (Pritchard et al, 1993; Pritchard et al, 1994). The GBS hyaluronate lyase, originally believed to be a neuraminidase (Milligan et al, 1978; Pritchard et al, 1993), is produced in high amounts by GBS isolated from infected infants (Milligan et al, 1978). Of all the GBS serotypes, type III strains were most frequently high producers of hyaluronate lyase (Milligan et al, 1978; Kjemis et al, 1980). Although the exact role of the GBS hyaluronate lyase has not been elucidated, Spellerberg (2000) proposes that it might play a role in allowing GBS to pass through the amniotic membrane as well as degrade cellular basement membrane to facilitate dissemination to other sites.

C protein

Between 40 and 60% of GBS strains have C protein antigens on their surface (Johnson et al, 1984; Hickman et al, 1999). Serotypes Ia, Ib, and II most frequently carry C protein antigens, while serotype III GBS rarely do. The GBS C protein antigens, designated α and β , can elicit a protective response in mice immunized with either of the antigens and challenged with C protein-carrying GBS (Michel et al, 1991; Madoff et al,

1992). However, deletion of the typical 9 repeats of 82 amino acids in the α -C protein alters the protein's immunogenicity rendering it unrecognizable by antibody (Madoff et al, 1996; Gravekamp et al, 1998). This type of antigenic variation is also seen with the Rib protein, a α -C protein-like surface molecule found primarily on serotype III GBS (Stalhammar-Carlemalm et al, 1993; Wastfelt et al, 1996).

The β -C component is a unique protein that functions as an IgA receptor by binding the Fc portion of the antibody. The 130-kilodalton β -C protein has a long N-terminal signal sequence and a region in the C-terminus where every third residue is a proline (Jerlstrom et al, 1991; Heden et al, 1991). Jerlstrom et al (1996) identified a 73 amino acid region in the C-terminal portion of the β -C protein that is responsible for IgA binding. By binding IgA via the Fc portion of the antibody, GBS interferes with opsonophagocytosis and effective clearance.

Superoxide dismutase

In 1997 Gaillot and colleagues cloned the gene for a manganese-superoxide dismutase (*sodA*) from GBS. Initial experimental evidence suggested that *sodA* functioned in protection against superoxide radicals generated as part of normal GBS metabolic activities (Gaillot et al, 1997). *sodA* mutants created by allelic exchange are more susceptible to oxidative stress and macrophage killing as well as show decreased survival in the bloodstream and brains of mice following intraperitoneal injection (Poyart et al, 2001). These results support a role for *sodA* in the protection of GBS against host defenses.

Purpose and Specific Aims

The purpose of this study was to examine the interactions between various components of the human innate immune system and group B streptococcus and determine what role these interactions might have on the GBS colonization process. In general, immunity to GBS has been relatively underexplored and the mechanisms contributing to colonization are unknown. We hypothesized that interactions between GBS and the innate immune system may either contribute to the colonization process or fail to prevent it. The following aims were used to test this hypothesis.

Aim 1: To compare the phagocytic engulfment capacities of monocytes and granulocytes from pregnant and nonpregnant, GBS-colonized and noncolonized women in response to GBS. We examined the abilities of phagocytes from these groups of women to engulf FITC-labeled GBS after 20 and 45 minutes of incubation. The mean fluorescence intensity, which indicated the relative numbers of GBS engulfed, was measured by flow cytometry.

Aim 2: To determine the potential killing capacities of monocytes and granulocytes from pregnant and nonpregnant, GBS-colonized and noncolonized women in response to GBS. Intracellular superoxide production subsequent to GBS engulfment was determined in a flow cytometry based assay by measuring the increase in fluorescence following oxidation of a nonfluorescent dye to a fluorescent one in the presence of superoxide. Release of superoxide into the extracellular environment after GBS engulfment was measured in a colorimetric assay.

Aim 3: To determine the mannose binding lectin exon-1 and promoter allele types and serum concentrations in GBS-colonized and noncolonized women. Mannose binding

lectin exon-1 alleles were determined by restriction fragment length polymorphism analysis of PCR products of a 119bp region of the first exon of the gene. Mannose binding lectin promoter alleles at two polymorphic sites was determined by sequence specific oligonucleotide hybridization. Mannose binding lectin protein levels were measured by enzyme linked immunosorbant assay.

Aim 4: To determine the effects of cytokine exposure on GBS growth. GBS growth was measured by optical density at 600 nm every hour for an 8-hour period during which GBS was incubated with cytokine at concentrations of 0-100 ng/ml. Additional studies examined potential synergism effects on GBS growth between interleukin-1 β and interleukin-6, as well as the requirement of interleukin-6 biologic activity for its growth promoting effects.

Aim 5: To determine whether or not GBS may bind interleukin-6. The binding interaction between GBS and FITC-interleukin-6 was measured by flow cytometry. The interleukin-6 – GBS interaction was verified by pre-incubating the interleukin-6 with an anti-human interleukin-6 blocking monoclonal antibody prior to addition to the GBS.

Aim 6: To determine the effects of IL-6 exposure on GBS virulence factors. RT-PCR analysis was performed on cDNAs made from GBS incubated with and without interleukin-6 using primers specific for a capsular polysaccharide gene, a cytolysin/hemolysin gene, and the C5a peptidase gene. Real time PCR was performed to verify RT-PCR results.

Aim 7: To determine the effects of IL-6 exposure on global GBS gene expression. Random arbitrarily primed PCR was performed on GBS incubated with and without interleukin-6 exposure to identify genes with increased expression after interleukin-6

exposure. The enhanced expression of genes identified by random arbitrarily primed PCR was verified by real time PCR.

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CHAPTER 2. DIFFERENCES IN INNATE IMMUNOLOGIC RESPONSE TO GROUP B STREPTOCOCCUS BETWEEN COLONIZED AND NONCOLONIZED WOMEN

A paper published in *Infectious Diseases in Obstetrics and Gynecology*¹

Jennifer M. Smith², Rachel H. Respass³, David G. Chaffin³, Bryan Larsen⁴, and Susan H. Jackman²

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²Departments of Microbiology, Immunology, & Molecular Genetics and Obstetrics and Gynecology, Marshall University School of Medicine, Huntington, WV

³Department of Obstetrics and Gynecology, Marshall University School of Medicine, Huntington, WV

⁴Des Moines University Osteopathic Medical Center, Des Moines, IA

This chapter addresses specific aims 1 and 2 by comparing the phagocytic engulfment of GBS and the subsequent production of superoxide by phagocytes from GBS-colonized and noncolonized women. These studies were designed to determine whether or not interactions between the innate immune system and GBS differ in these groups of women. Such differences in immune responses might lead to the undermining of GBS clearance mechanisms and therefore contribute to the initiation of the colonization process.

ABSTRACT

Objective: To evaluate the functional capacity of granulocytes and monocytes from pregnant and nonpregnant women in relation to group B streptococcus (GBS) colonization status.

Methods: Engulfment of fluorescent GBS by peripheral blood phagocytes from GBS-colonized and noncolonized women was measured by flow cytometry. Intracellular superoxide generated in response to GBS challenge to monocytes and granulocytes

enriched from peripheral blood of these women was also measured by flow cytometry and extracellular superoxide was determined by colorimetric assay.

Results: Monocytes and granulocytes from pregnant, GBS-colonized women engulfed significantly greater numbers of GBS than phagocytes from pregnant, noncolonized women or nonpregnant women regardless of colonization status. No difference in intracellular superoxide production was detected between any of the groups of women, however monocytes from pregnant, colonized women released significantly more superoxide into the extracellular milieu than did granulocytes from the same women. No differences in extracellular release of superoxide were observed among noncolonized women whether they were pregnant or not.

Conclusions: Monocytes from pregnant, colonized women engulf more GBS and release more of the superoxide into the extracellular environment where it is unlikely to be an effective defense mechanism against intracellular bacteria. This suggests that components of the innate immune system that should serve in a protective role may function suboptimally thereby contributing to the colonization process by GBS.

KEY WORDS

phagocytosis; superoxide; flow cytometry

INTRODUCTION

Over the past thirty years group B streptococcus (GBS) has gained prominence as the primary etiologic agent of sepsis, bacteremia, and meningitis in the neonatal period. Administration of intrapartum antibiotics to women identified at risk for transmitting GBS can be effective in prevention of transmission and early-onset disease¹, but it fails to address the underlying problem of maternal colonization. Despite estimates that between

15 and 35% of women are asymptotically colonized with GBS² little is known as to why GBS can evade the normal immune response in some women (colonized) and not in others (noncolonized).

Phagocytic cells, particularly monocytes and granulocytes, are key mediators of innate immunity to microorganisms, providing an early, rapid response to a potential pathogen while the subsequent antigen-specific response is being initiated. Phagocytosis of an organism stimulates production of microbicidal reactive oxygen species, including superoxide radicals and hydrogen peroxide. Microbial degradation products generated from the combined effects of the respiratory burst and cytolytic enzymes are then processed and presented to induce either a T cell- or antibody-mediated response. Alterations of these early steps in defense can lead to ineffective clearance of the microorganism. While extreme examples of these defects such as leukocyte adhesion deficiencies,³ or respiratory burst defects (chronic granulomatous disease)⁴ result in severe, chronic bacterial and fungal infections, more subtle changes due to the unique physiologic adaptations of pregnancy may be involved in the infectious conditions that are particular threats to mothers and their newborns.

To evaluate the role that alterations in early innate immune responses to GBS may play in supporting the colonization process, the phagocytic and respiratory burst capacities of GBS-colonized and noncolonized women were compared. Because GBS colonization is primarily an obstetric concern and the hormonal changes of pregnancy influence immunologic functioning, comparisons were also made between pregnant and nonpregnant women irrespective of colonization status.

In this study we show that while pregnant women display an increased engulfment of GBS, superoxide release from the phagocytic cells may be increased, providing a possible explanation for colonization and modified adaptive immune response in pregnancy.

SUBJECTS AND METHODS

Study Population

The populations studied consisted of pregnant and nonpregnant women recruited from Marshall University School of Medicine and its associated obstetric clinics. All patients were at least 18 years of age, had no other known active infections or immunodeficiency, and were not taking antibiotics in the previous six weeks. The study was approved by the Marshall University School of Medicine Institutional Review Board and each woman gave informed consent prior to participation. Peripheral blood from each participant was drawn from the antecubital vein into EDTA Vacutainer tubes. Amies clear StarSwabs were used to collect specimens for determination of GBS colonization status. Swabs of the lower third of the vagina from pregnant patients were sent to the Cabell Huntington Hospital laboratory as part of routine obstetric screening. Self-collected swabs from the nonpregnant patients were taken to the research laboratory. All swabs were incubated in Lim broth for twelve hours at 37°C in 5%CO₂. Aliquots of the Lim broth culture were streaked onto tryptic soy agar with 5% sheep blood and incubated overnight at 37°C in 5% CO₂. Presumptive identification of GBS was based on colony morphology, hippurate catalysis, and CAMP test (research laboratory only). Colonies were confirmed as group B streptococcus by latex agglutination (Meritec Strep Group B

Beta-hemolytic Streptococcus grouping set; Meridian Diagnostics, Inc., Cincinnati, OH) by both laboratories.

Based on pregnancy status and the results from the vaginal swab cultures the women were placed into four groups: pregnant, GBS-colonized (n = 9 for phagocytic assays; n = 5 for superoxide assays), pregnant, noncolonized (n = 8 for phagocytic assays; n = 5 for superoxide assays), nonpregnant, GBS-colonized (n = 3 for all assays), and nonpregnant, noncolonized (n = 3 for all assays). For the pregnant, GBS-colonized women the mean age was 26.5 years, gravidity ranged from 1 to 7, and parity ranged from 0 to 4. For the pregnant, noncolonized women the mean age was 23.1 years, gravidity ranged from 1 to 6, and parity ranged from 0 to 2. For the nonpregnant, GBS-colonized women the mean age was 38.7 years and gravidity and parity were both 0. For the nonpregnant, noncolonized women the mean age was 30.3 years and gravidity and parity were both 0. All women were Caucasian with the exception of one pregnant, noncolonized woman who was of American Indian heritage. All women were healthy volunteers with medical insurance and with an estimated annual family income of greater than \$15,000.

Bacterial Preparation

Group B streptococci isolated from asymptomatic, colonized women during routine gynecologic exams were assigned the designations 2118 and 2407 corresponding to randomly assigned patient identification numbers. GBS 2118 and 2407 were serotyped as type V and type III, respectively, using a hemolytic streptococcus group B typing sera kit (Accurate Chemical & Scientific Corp., Westbury, NY). GBS 2118 was

used for the phagocytic engulfment assays due to poor fluorescence labeling of the type III isolates like GBS 2407 used in the superoxide assays.

For the phagocytic engulfment assays GBS 2118 was labeled by incubation with fluorescein isothiocyanate (FITC) 1 hydrochloride (Research Organics, Cleveland, OH) at 50µg/ml in Todd-Hewitt broth for 16 hours at 37°C in 5% CO₂. The cultures were centrifuged, washed twice with phosphate-buffered saline (PBS), and heat-killed at 60°C for 45 minutes prior to resuspension to a final concentration of 10⁸ bacteria/ml. After confirming staining via flow cytometry (FACScan, Becton Dickinson, San Jose, CA) the FITC-GBS were aliquoted and stored at -70°C until needed.

For the superoxide assays GBS 2407 was inoculated from frozen stocks and cultured in Todd-Hewitt broth at 37°C in 5% CO₂ until an optical density at 600nm (OD₆₀₀) of 0.4 was reached (approximately 4 hours).

Phagocytic Engulfment

Plasma was removed from 100µl of whole blood. The cell pellets were washed twice in Dulbecco's phosphate-buffered saline (DPBS) and resuspended in 100µl heat-inactivated pooled human type AB serum (Sigma, St. Louis, MO). The pooled human type AB serum failed to agglutinate GBS 2118 in a slide agglutination test. FITC-GBS 2118 was sonicated briefly to create a single-cell suspension prior to adding 10µl to each tube of resuspended leukocytes. After incubation at 37°C on a rotary shaker for the appropriate length of time, cytochalasin D (Sigma, St. Louis, MO) was added to each tube (5µM final concentration). The contents of each tube were diluted with ice-cold DPBS, centrifuged, and washed twice with cold DPBS. Erythrocytes were lysed using ImmunoLyse (Coulter, Miami, FL) according to the manufacturer's instructions. The

leukocyte pellet was washed twice with DPBS and the cells were stained for CD14(phycoerythrin-labelled clone TUK1; Caltag Laboratories, Burlingame, CA) to allow for differentiation of leukocyte populations using flow cytometry. Stained cells were fixed in 4% paraformaldehyde and analyzed by flow cytometry. Side light scatter properties and CD14 staining were used to discriminate between monocytes and granulocytes. A minimum of 5000 events were collected for each population and analyzed for the percentage of cells having engulfed FITC-GBS and for mean fluorescence intensity using CellQuest Software (Becton Dickinson).

Superoxide Assays

Enriched populations of monocytes and granulocytes were used for measurements of superoxide generated subsequent to engulfment of GBS 2407. Monocytes were enriched from whole peripheral blood by density gradient centrifugation using Ficoll Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ) followed by negative selection using the StemSep human monocyte enrichment system (Stem Cell Technologies, Vancouver, British Columbia, Canada). Granulocytes were enriched from the erythrocyte-granulocyte pellet from the density gradient centrifugation by dextran sedimentation (500,000 average molecular weight; Sigma) followed by hypotonic saline erythrocyte lysis. Viability was assessed by trypan blue staining and a minimum viability of 90% was required for use in any assay. Purity of the enriched monocyte populations was demonstrated using flow cytometry. Pure monocytes were positive for CD14 and negative for CD3 (clone UCHT1; BD PharMingen, San Diego, CA) and CD19 (clone HIB19; BD PharMingen). Purity of the enriched granulocyte populations was demonstrated using forward and side light scatter properties via flow cytometry.

One hundred microliter aliquots of enriched monocytes or granulocytes (1×10^6 cells/ml) in PBS-gel were loaded with hydroethidine ($10\mu\text{M}$ final concentration; Polysciences Inc., Warrington, PA) for 15 minutes at 37°C . The cells were stimulated by the addition of either phorbol 12-myristate 13-acetate (PMA) at a 200ng/ml final concentration or $10\mu\text{l}$ of log-phase GBS 2407. The fluorescence level was immediately determined by flow cytometry and was used as the zero time point. The granulocytes and monocytes were incubated for 20 minutes and 45 minutes, respectively, at 37°C with the stimulators before the final fluorescence reading was taken. A minimum of 5000 events were collected for each population and analyzed for the percentage of cells with an increase in fluorescence over time after stimulation.

In order to measure superoxide released from the monocytes and granulocytes $100\mu\text{l}$ aliquots of cells ($1 \times 10^6/\text{ml}$) in PBS-gel were incubated for 45 minutes at 37°C in 5% CO_2 with $25\mu\text{l}$ cytochrome c (2.7mg/ml ; from horse heart), in the presence ($25\mu\text{l}$) or absence of superoxide dismutase (1mg/ml ; SOD; from bovine erythrocytes), and with either 200ng/ml PMA or $10\mu\text{l}$ of log phase GBS 2407. Monocyte- and granulocyte-free tubes were prepared as controls. After incubation the tubes were centrifuged and the supernatants were transferred to a 96-well plate. The absorbance at 550nm was read and used to calculate the nanomoles of superoxide released according to the following formula: (experimental – matched cell-free control) – (experimental with SOD – matched cell-free control) $\div 47.4$.

Statistical Analysis

Statistical analyses were performed using the SigmaStat software package (Jandel Scientific Software, San Rafael, CA). The Student's *t*-test was used for comparisons of

pregnant versus nonpregnant women and colonized versus noncolonized women. $P < 0.05$ was considered statistically significant. The t -test sample size program (alpha = 0.05 and power = 0.8) was used to estimate the number of individuals needed to provide statistical significance when $P > 0.05$.

RESULTS

Phagocytic Engulfment

The percentages of monocytes and granulocytes having engulfed FITC-GBS 2118 were measured by flow cytometry and compared between GBS-colonized and noncolonized women. No significant differences were observed between these groups of women regardless of pregnancy status (TABLE 1).

Although no differences in the percentages of phagocytes having engulfed FITC-GBS 2118 were observed, measurements of mean fluorescence intensity revealed a significant difference in the relative number of FITC-GBS 2118 taken up by phagocytes from pregnant, colonized women when compared to pregnant, noncolonized women. Granulocytes from pregnant, colonized women engulfed approximately twice the number of FITC-GBS 2118 after 20 and 45 minutes that those from pregnant, noncolonized women (Fig. 1A). Monocytes from pregnant, colonized women engulfed greater than five times the number of FITC-GBS 2118 after 45 minutes than those from pregnant, noncolonized women (Fig. 1A). These differences were not observed for nonpregnant women (Fig. 1B) or in parallel experiments substituting *Staphylococcus aureus* (ATCC 25923) for GBS 2118 (data not shown).

Superoxide Assays

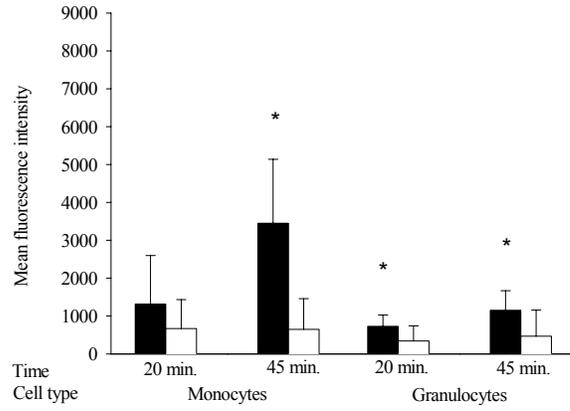
TABLE 1. Percentages^a of phagocytes having engulfed FITC-GBS 2118 over time

Pregnancy Status	Colonization Status	Cell Type	% Phagocytes Containing GBS	
			20 min.	45 min.
Pregnant	Colonized (n = 9)	Monocytes	55.9 ± 7.8	69.1 ± 12.8
		Granulocytes	58.6 ± 18.6	79.1 ± 14.3
	Noncolonized (n = 8)	Monocytes	34.7 ± 7.8	42.0 ± 19.2
		Granulocytes	29.4 ± 11.4	40.7 ± 18.7
Nonpregnant	Colonized (n = 3)	Monocytes	48.4 ± 14.9	55.5 ± 14.1
		Granulocytes	52.3 ± 21.2	67.3 ± 19.4
	Noncolonized (n = 3)	Monocytes	37.4 ± 15.5	44.1 ± 11.6
		Granulocytes	25.6 ± 9.8	37.1 ± 30.0

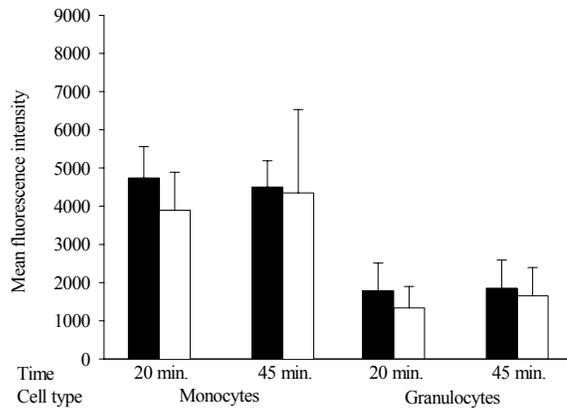
^aMeans ± standard deviation.

Fig. 1. Mean fluorescence intensity of engulfed FITC-GBS 2118 over time. Monocytes and granulocytes from colonized (black bars) and noncolonized (white bars), pregnant (A) and nonpregnant (B) women were incubated with FITC-GBS 2118 for 45 min. Mean fluorescence intensity was measured using flow cytometry. Significant differences between colonized and noncolonized women are denoted by asterisks ($p < 0.05$ using Student's *t*-test). Pregnant, colonized: $n = 9$; pregnant, noncolonized: $n = 8$; nonpregnant, colonized: $n = 3$; nonpregnant, noncolonized: $n = 3$.

A



B



Preliminary experiments were conducted to determine the time and PMA concentration required for optimal superoxide production. The concentrations and time superoxide (0.78 ± 0.8 nmol) into the extracellular environment than did the granulocytes points used in both superoxide assays reflected maximal PMA as well as GBS stimulation.

A comparison of the percentages of enriched monocytes and granulocytes producing intracellular superoxide in response to challenge with log-phase GBS 2407 failed to show significant differences between GBS-colonized and noncolonized women regardless of pregnancy status (Fig. 2).

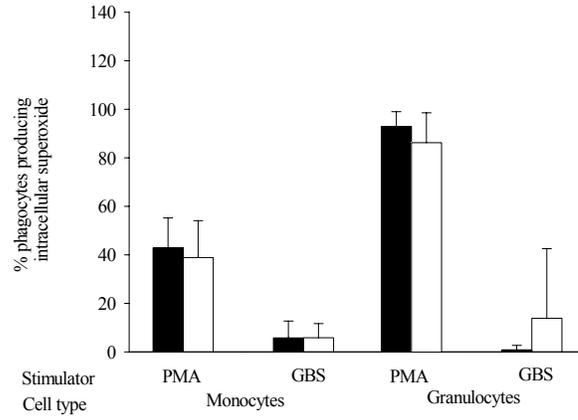
Not all superoxide generated intracellularly remains as such. Extracellular release of superoxide by enriched monocytes and granulocytes in response to challenge with log-phase GBS 2407 was measured spectrophotometrically after 45 minutes. No significant differences between colonized and noncolonized women were detected. However, monocytes from pregnant, colonized women released a significantly greater amount of from these same women (0.0 ± 0.0 nmol) (Fig. 3A). This difference was not observed for noncolonized women regardless of pregnancy status (Fig. 3A, B). Parallel experiments with *S. aureus* 25923 again demonstrated the specificity of these differences in relation to GBS (data not shown).

DISCUSSION

Despite recent efforts to devise more accurate and cost-effective methods for identifying and treating pregnant women at risk of transmitting GBS to their newborns much remains to be learned concerning basic immunity to GBS. Perhaps the most important issue in this area is why certain women become colonized with GBS while

Fig. 2. Generation of intracellular superoxide after stimulation with PMA or GBS 2407. Enriched populations of monocytes and granulocytes from colonized (black bars) and noncolonized (white bars), pregnant (A) and nonpregnant (B) women were loaded with hydroethidine prior to incubation at 37°C with PMA (200ng/ml) or log-phase GBS 2407. The increase in fluorescence due to superoxide oxidation of hydroethidine to ethidium bromide was measured using flow cytometry at 20 minutes (granulocytes) and 45 minutes (monocytes) after stimulation. No significant differences among any of the groups were observed. Pregnant, colonized and noncolonized: n = 5; nonpregnant, noncolonized: n = 3.

A



B

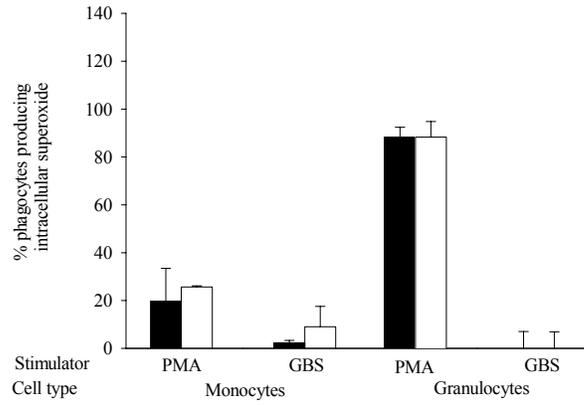
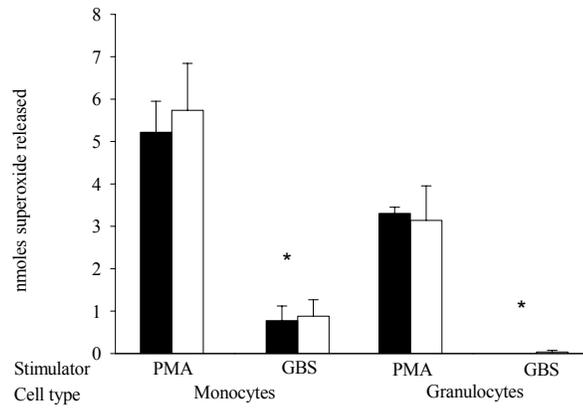
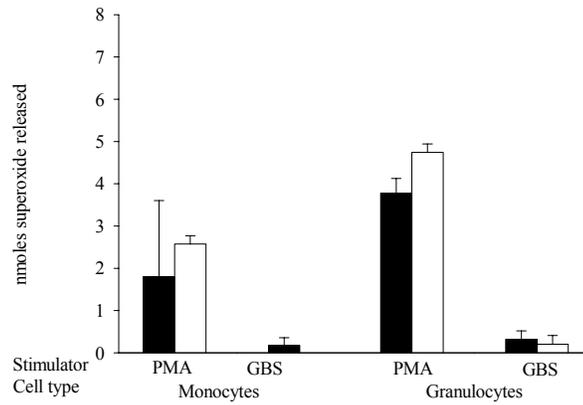


Fig. 3. Release of superoxide into the extracellular milieu following stimulation with PMA or GBS 2407. Enriched populations of monocytes and granulocytes from colonized (black bars) and noncolonized (white bars), pregnant (A) and nonpregnant (B) women were stimulated with PMA (200ng/ml) or log-phase GBS 2407 for 45 minutes at 37°C. The nanomoles of superoxide released were calculated from changes in absorbance at 550nm. Significant differences between monocytes and granulocytes from pregnant, colonized women are denoted by asterisks ($p < 0.05$ using Student's *t*-test). Pregnant, colonized and noncolonized: $n = 5$; nonpregnant, colonized and noncolonized: $n = 3$.

A



B



others effectively eliminate it. We have proposed that the way the innate immune system in GBS-colonized women responds to GBS differs from that in noncolonized women and that these differences may assist in the initiation of colonization. To test this proposal we compared the abilities of phagocytes from colonized and noncolonized women to engulf GBS and generate the microbicidal superoxide radical following engulfment. Possible contributions due to pregnancy were also evaluated by comparing responses from pregnant and nonpregnant women.

While no differences in the number of phagocytes having engulfed GBS were observed for any of the groups of women, the bacterial load in phagocytes from pregnant, colonized women was significantly greater than for pregnant, noncolonized women. This effect was probably not due to opsonic antibody since heterologous serum that failed to agglutinate GBS was used in these assays. Both granulocytes and monocytes from pregnant, GBS-colonized women engulfed greater numbers of GBS. Although overall enhanced phagocytic ability is frequently observed during pregnancy^{5,6,7} the greater than five-fold increase in engulfment by monocytes and the two-fold increase in engulfment by granulocytes from pregnant, GBS-colonized women appears to be specific to GBS as these differences were not observed in parallel experiments with *S. aureus*.

Following phagocytic engulfment of GBS, superoxide is generated within the phagocyte by phagosome-associated NADPH oxidase. Not surprisingly, differences with regard to the percentage of phagocytes producing superoxide were not seen, but there was a difference with respect to pregnant, GBS-colonized women in the amount of superoxide released from monocytes versus granulocytes. The release of superoxide into the extracellular environment was greater from monocytes in pregnant, GBS-colonized

women. Granulocytes from these women failed to release any superoxide extracellularly.

The mechanism underlying the release of superoxide generated within the phagosome into the extracellular environment is unclear. Two possible explanations can be envisioned, one phagocyte-associated and one GBS-associated. The respiratory burst associated with phagocytic engulfment is activated following specific microbe-phagocyte receptor interactions. If the respiratory burst is activated through these interactions prior to complete closure of the phagosome, superoxide could leak into the extracellular environment. Alternatively, factors produced by GBS may break down the phagocyte membrane integrity leading to leakage of superoxide outside the cell. Studies have shown that GBS can cross various types of eukaryotic cell membranes including those from human chorion cells,⁸ human umbilical vein endothelial cells,⁹ human lung epithelial cells,¹⁰ and human monocytes.¹¹ Using GBS serotype III strains producing different levels of β -hemolytic activity^{9,10} or using agents that inhibit GBS β -hemolysin¹¹ it has been demonstrated that the β -hemolysin produced by GBS disrupts cellular membranes, alters membrane permeability, and causes the release of lactate dehydrogenase. Based on the findings of these studies it can be argued that the β -hemolysin produced by GBS 2407, the serotype III isolate used in our superoxide assays, may damage the plasma and/or phagosome membranes leading to a release of superoxide into the extracellular environment. Both of these possible mechanisms for the release of superoxide are subjects for future investigations, perhaps by using strains with a deletion of the β -hemolysin gene.

Although a number of studies have investigated the interactions between GBS and phagocytes, only one employs flow cytometry to measure phagocytic engulfment and

superoxide production.¹² In that study the authors report that phagocytosis of GBS and superoxide production after ingestion are greater for neutrophils than for monocytes, but does not evaluate the effect of pregnancy which may explain the differences with our study. In addition to differences in study populations, McCloskey and Salo¹² uses GBS incubated with antibody and complement to examine opsonophagocytosis. In contrast our study used unopsonized GBS to examine engulfment.

In that colonization status, as well as pregnancy, defined our study populations we were aware that differences in sample collection techniques would inevitably lead to variations in GBS recovery rates. We elected to allow the physician to determine the collection procedure. As only lower vaginal segment GBS colonization was examined in our study, GBS carriage may have been modestly underestimated. However, statistical sample size evaluation suggested that the addition of more subjects was unlikely to alter the conclusions of the study.

The results of our investigations indicated a unique interaction between GBS and monocytes from pregnant, GBS-colonized women. GBS may accumulate in large numbers in monocytes from colonized, pregnant women, which tend to release their superoxide into the extracellular milieu. Superoxide is most likely to be more potent when sequestered in an intracellular location therefore, these monocytes, which have released their superoxide, may in effect create an intracellular niche for GBS. Other recent studies support the idea that monocytes provide an intracellular haven for GBS. Several groups have reported that GBS can persist intracellularly in murine macrophages for 24 to 48 hours following infection^{13,14} as well as inactivate the protein kinase C

pathway, one of the main signal transduction pathways leading to macrophage activation.¹⁴

The alterations in innate immune response by monocytes from pregnant, colonized women demonstrated here provide the initial evidence that the immune system, which should function in a protective fashion, may not contribute to the prevention of GBS colonization. These alterations are by no means the only contributory factors in the colonization process, but provide a framework for further dissection of the underlying mechanism. In the process of elucidating the mechanism leading to GBS colonization more information on the immune response to GBS will be generated and may provide a clearer path to the design of an effective GBS vaccine.

ACKNOWLEDGEMENTS

We thank Sandra White for her assistance in obtaining the blood samples from the pregnant participants and Pamela Staton for her assistance in obtaining blood samples from the nonpregnant participants.

These studies were presented in part at the International Infectious Disease Society for Obstetrics and Gynecology – USA Fifth Annual Conference on Women’s Health held May 2000 in San Francisco, CA.

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CHAPTER 3. ELEVATED SERUM MANNOSE BINDING LECTIN IN GROUP B STREPTOCOCCUS COLONIZED WOMEN

A paper to be submitted to the *Journal of Infectious Diseases*.

Jennifer M. Smith¹, Xuejun Peng², Stephen K. Hunter³, and Susan H. Jackman¹

¹Departments of Microbiology, Immunology, & Molecular Genetics and Obstetrics & Gynecology, Marshall University Joan C. Edwards School of Medicine, Huntington, WV

²Department of Statistics, University of Kentucky, Lexington, KY

³Department of Obstetrics and Gynecology, Division of Maternal-Fetal Medicine, University of Iowa Hospitals & Clinics, Iowa City, IA

Results from the studies presented in the previous chapter demonstrated that there is enhanced internalization of GBS by phagocytes from pregnant, GBS-colonized women in comparison to noncolonized women. This increased engulfment may be mediated in part by opsonization with GBS-specific antibody, complement, or lectins. We know from the design of the previous experiments that antibody and complement were not potential opsonins under those conditions. Therefore we chose to investigate the role one of the lectins, mannose binding lectin, may play in phagocytosis of GBS. These experiments addressed specific aim 3.

ABSTRACT

Mannose binding lectin (MBL) gene polymorphisms and serum concentrations were examined in GBS-colonized and noncolonized women. PCR and restriction fragment length polymorphism analysis was used to identify individuals with variant alleles for exon-1 of the MBL gene. Sequence-specific oligonucleotide hybridization was used to identify individuals with polymorphisms at two loci in the MBL gene promoter region. Serum MBL concentrations were determined by ELISA. No associations were observed between GBS colonization status and MBL gene polymorphisms. However, ELISA data indicated that there was an increased serum level of MBL in GBS-colonized

women compared to noncolonized women ($P = 0.0114$). In a flow cytometry-based phagocytosis assay, increased serum MBL levels were associated with increased engulfment ($P < 0.05$). This association between serum MBL levels, GBS colonization, and increased phagocytosis suggests that MBL may be involved in enhanced engulfment, which leads to the creation of an intracellular niche and persistent colonization.

INTRODUCTION

Group B streptococcus (GBS) has emerged as the most prevalent cause of pneumonia, sepsis, and meningitis during the neonatal period. The majority of life-threatening GBS infections are transmitted from a colonized mother to her infant during the birthing process. As such, maternal colonization in the vagina and/or rectum is a major risk factor for neonatal GBS disease. In the United States this puts 15-35% of the female population in danger of passing GBS to the newborn [1]. In spite of such a large group at risk, little is understood concerning the events contributing to persistent colonization with GBS in some women.

The collectin family of serum proteins function as mediators in the nonspecific immune response by binding surface carbohydrates of microorganisms. This collectin-microbe interaction enhances phagocytosis and microbial clearance. In addition to acting directly as an opsonin [2], mannose binding lectin (MBL) is capable of activating both the classical [3] and alternative [4] complement cascades. The importance of the role MBL plays in host immune responses is underscored by numerous studies examining the relationships between MBL and infectious and inflammatory diseases. Deficiencies in MBL due to polymorphisms in the promoter and mutations in exon-1 of this gene are associated with susceptibility to infections by a variety of microorganisms, including *N.*

meningitidis [5], *P. falciparum* [6], and *C. neoformans* [7]. However, with some intracellular microbes, like *L. chagasi* [8], MBL deficiency may protect against infection by blocking entry into host cells.

Because MBL functions to enhance microbial engulfment by host cells, we examined the relationship between mannose binding lectin and GBS colonization. Our data did not demonstrate a relationship between any of the MBL gene mutations and polymorphisms associated with MBL serum protein deficiency and GBS colonization. In fact, significantly higher levels of serum MBL protein were observed in women colonized with GBS. Additionally, at high serum concentrations of MBL, engulfment of GBS by phagocytes was significantly increased.

MATERIALS AND METHODS

Subjects. Sixty-eight reproductive age women were recruited from Marshall University Joan C. Edwards School of Medicine, Huntington, WV, and an obstetric and gynecology clinic at the University of Iowa, Iowa City, IA. Participants were grouped as GBS-colonized or noncolonized according to results of cultures from ano-vaginal swabs. Racial composition, socioeconomic status, age, gravidity, and parity were similar for both groups.

DNA Isolation from Blood Spots. Blood from EDTA Vacutainer tubes or fingersticks were spotted onto Whatman filter paper or FTA Cards (Life Technologies, Rockville, MD). Blood spots on filter paper were fixed in methanol and air-dried while those on FTA Cards were allowed to air dry over night. Two 2 mm disks were removed from the dried blood spots. DNA was released from the filter paper disks by boiling for 10

minutes in sterile, deionized water and from the FTA cards using FTA Extraction Reagent (Life Technologies).

MBL Exon-1 and RFLP. DNA extracts were used as the template in PCR reactions and subsequent RFLP analysis as previously described [9]. Briefly, a 119-bp region of the first exon of the MBL gene was amplified by PCR and then digested by *Ban I*, *Mbo II*, or *Mlu I*. Restriction digestion banding patterns were used to identify the alleles as either wild-type or B, C, and D variants (See Appendix B, Figure 1).

MBL Promoter PCR and Sequence-specific Oligonucleotide (SSO) Hybridization. The MBL gene promoter from -801 to +77 was amplified from DNA extracted from blood spots in PCR reactions as described previously [10]. The PCR product was transferred to a nylon membrane by slot blot. MBL promoter allele SSO probes labeled using the BrightStar Psoralen-Biotin Nonisotopic Labeling Kit (Ambion, Austin, TX) were hybridized to the slot blot in TMAC hybridization buffer (50 mM Tris, pH 8; 3 M tetramethylammonium chloride; 2 mM EDTA; 0.1% SDS; 5X Denhardt's solution; 100 µg/ml herring sperm DNA) for 3 hours with gentle shaking at 54°C. The membranes were then washed twice with 2X SSPE (3 M NaCl; 20 mM NaH₂PO₄; 2 mM EDTA, pH 7.4) at room temperature and twice with TMAC at 59°C. Probe hybridization was detected using the BrightStar BioDetect Nonisotopic Detection Kit (Ambion).

MBL Serum ELISA. Serum was collected in Vacutainer tubes without anticoagulant and frozen at -20°C until use. Nunc MaxiSorp microplates (Fisher Scientific, Pittsburgh, PA) were coated with 1 µg/ml mouse anti-human mannan binding lectin monoclonal antibody clone HYB131-01 (Statens Serum Institute, Copenhagen, Denmark) overnight at 4°C. The plates were blocked with PBS-Tween 20 (PBS-T) for two hours at room temperature.

Serum samples were diluted 1:10 in PBS-T and aliquoted into duplicate wells. MBL standard and deficient sera (Statens Serum Institute) were diluted 1:10 and then serially diluted at 1:2 in PBS-T. The plate was incubated overnight at 4°C. After washes with PBS-T, 0.5 µg/ml biotinylated HYB131-01 (Statens Serum Institute) was added and the plate was incubated for one hour at room temperature. Following washes with PBS-T, horseradish peroxidase avidin D (Vector, Burlingame, CA) was added to each well and incubated for 30 minutes at room temperature. Following washes 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Vector) was added and color was allowed to develop for 30 minutes after which the absorbance was measured at 504 nm. A standard curve was created from MBL standard serum samples by linear regression using SigmaStat 2.03 software (Jandel Scientific, San Rafael, CA). Serum MBL concentrations of test samples were calculated using the regression equation and reported in ng/ml.

Phagocytic Engulfment. Phagocytic engulfment was measured as described previously [11] with minor modification. The cell pellet from a small aliquot of whole blood was resuspended in two-fold serial dilutions of MBL standard serum (in PBS with 5 mM CaCl₂) prior to incubation with FITC-GBS for 45 minutes at 37°C. After erythrocyte lysis and fixation in 2% paraformaldehyde, flow cytometry was used to analyze the percentage of phagocytes having engulfed FITC-GBS.

Statistics. The association between MBL gene polymorphisms and GBS colonization was analyzed by either Chi-squared or Fisher's Exact test according to the number of observations in each category. Wilcoxon rank sum test or Kruskal-Wallis test was used to analyze the difference in MBL serum concentrations between MBL gene polymorphisms and GBS colonization. Percentages of phagocytes having engulfed GBS

at different serum MBL concentrations were compared by one-way analysis of variance with the Tukey test for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Due to the innate immune functions attributed to mannose binding lectin and the association between MBL deficiency and infectious disease, MBL exon-1 alleles were examined by PCR and RFLP analysis in GBS-colonized and noncolonized women. Exon-1 alleles were identified as wild type or B, C, and D variants according to the restriction digestion banding pattern (See Appendix B, Figure 1). Seventeen GBS-colonized and thirty noncolonized women carried the wild-type alleles (table 1). Of the 12 GBS-colonized women with variant exon-1 alleles, 10 were heterozygous for the B allele and 2 were heterozygous for the D allele. Of the 9 noncolonized women with variant alleles, 5 were heterozygous for the B allele, 3 were heterozygous for the D allele, and 1 was heterozygous with both the B and D alleles. In both groups of women B and D allele homozygotes and C variant alleles were not observed. A relationship between MBL exon-1 alleles and GBS colonization was not detected ($\chi^2 = 0.1062$).

Since GBS colonization was not associated with variant exon-1 alleles, polymorphisms at two sites within the MBL gene promoter were analyzed using SSO hybridization in 26 of the GBS-colonized women and 24 of the noncolonized women. Alleles were identified as either L or H at the -550 polymorphic site and as either X or Y at the -221 polymorphic site in the MBL promoter region. Association between these polymorphisms at the -550 or -221 promoter sites and GBS colonization was also not detected (table 1).

Table 1. Relationship between MBL alleles and GBS colonization.

Colonization Status	Exon-1 Allele Type		Promoter Allele Type					
	Wild-type	Variant ^a	-550 site ^b			-221 site ^c		
			LH	LL	HH	XX	XY	YY
GBS +	17	12	15	11	0	1	23	2
GBS -	30	9	12	12	0	4	20	0

^aVariant refers to the number of individuals heterozygous for any variant allele.

^bThe -550 polymorphic site in the MBL promoter has alleles designated L and H. The values below represent the number of women with the specified allele combination at that site.

^cThe -221 polymorphic site in the MBL promoter has alleles designated X and Y. The values below represent the number of women with the specified allele combination at that site.

In addition to examining a potential relationship between MBL and GBS colonization at the genetic level, serum MBL protein concentrations were measured by ELISA. When all subjects were considered together, an association between women with the wild-type exon-1 alleles and higher levels of MBL was observed ($P = 0.048$; See Appendix B, Figure 2) which is similar to reports from others [9]. Interestingly, when comparing GBS-colonized versus noncolonized women, serum MBL protein quantities were significantly elevated in GBS-colonized women ($P = 0.0114$) as illustrated in figure 1A.

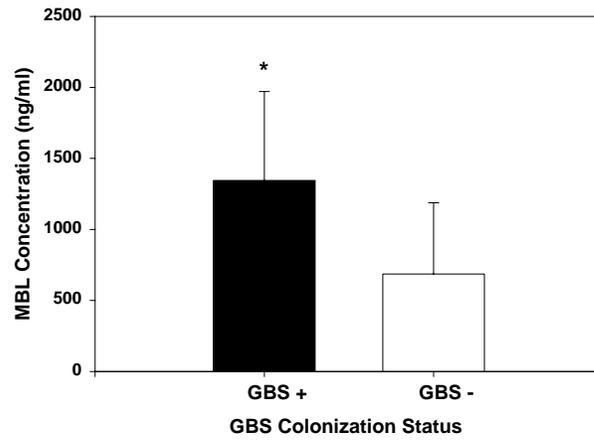
In order to evaluate the role MBL may play in phagocytic engulfment of GBS, a flow cytometry-based assay using serum with a known MBL concentration as a source of opsonin and peripheral blood phagocytes from normal, healthy volunteers was performed. Engulfment of GBS by phagocytes in the presence of MBL serum concentrations ranging from 3340 ng/ml to 104 ng/ml was measured. Figure 1B shows that at high concentrations of MBL internalization of GBS is significantly increased ($P < 0.05$), while lower concentrations (<1670 ng/ml) did not appear to enhance phagocytosis.

DISCUSSION

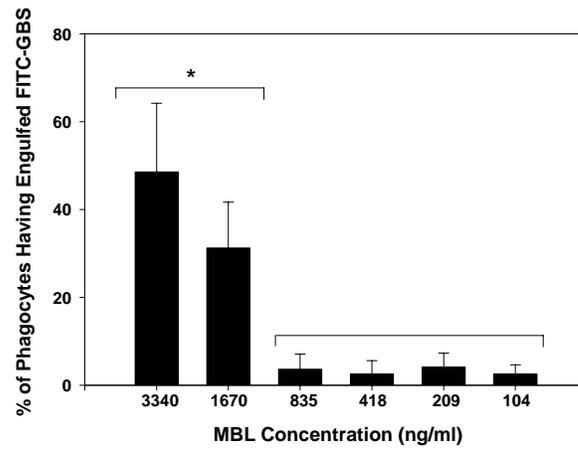
Studies linking deficiencies in serum mannose binding lectin protein with susceptibility to a multitude of infections emphasize the role of MBL as a mediator of innate immunity. Because of its demonstrated importance in host defense, we examined the possibility that a deficiency in MBL was associated with GBS colonization. Our initial data examining the frequencies of the MBL gene alleles associated with MBL deficiency indicated a strong association between variant exon-1 alleles and GBS

Figure 1. Associations between serum MBL concentration, GBS colonization status, and phagocytic engulfment. A. Increased serum MBL concentration in GBS-colonized women (n = 19) compared to noncolonized women (n = 11). $P = 0.0114$. B. Phagocytic engulfment of GBS is dependent on serum MBL concentration. Asterisk indicates $P < 0.05$ when comparing to MBL concentration of ≥ 1670 ng/ml to concentrations ≤ 1670 ng/ml.

A



B



colonization [12]. However, with the inclusion of more subjects, the significance of this relationship was reduced and, additionally, no correlation between the MBL promoter polymorphisms and colonization was observed (table 1). Finding no genetic association between MBL and GBS colonization status, we expected that MBL serum concentrations would be similar in both GBS-colonized and noncolonized women. However, the data revealed that GBS-colonized women had significantly higher levels of serum MBL than did noncolonized women (figure 1A). From these findings the question arose as to whether MBL assists in establishing persistent GBS colonization in some women. Using a phagocytic engulfment assay, we found that enhanced uptake of GBS was observed at increased serum levels of MBL.

The data presented here demonstrate that both enhanced GBS engulfment and GBS colonization status were associated with increased serum MBL levels. This supports our previous study showing that phagocytes from pregnant, colonized women engulf significantly greater numbers of GBS than pregnant, noncolonized women [11]. These data suggest that MBL may act to augment the creation of a protective niche for GBS within the phagocyte since others [13, 14] have indicated that upon engulfment by murine macrophages GBS survive intracellularly for 24-48 hours.

Similar to our findings, De Miranda Santos et al [8] report that there is a strong association between MBL serum concentrations and susceptibility to visceral leishmaniasis. High levels of serum MBL (2888 ng/ml) were observed in patients with a history of visceral leishmaniasis and low levels (944 ng/ml) were observed in individuals with negative leishmanin skin tests [8]. Perhaps most interestingly, asymptomatic individuals with a positive leishmanin skin test had intermediate serum MBL

concentrations (1113 ng/ml) [8], which are similar to the levels recorded for our asymptomatic GBS-colonized women (1344 ng/ml).

Our results suggest that MBL may function to enhance engulfment of GBS in colonized women, which at first glance would appear to conflict with previous data by Butko et al [15] suggesting that MBL is not an opsonin for serotype III GBS. Two important differences exist between our research design and that of Butko et al: 1) in Butko et al, the study population is composed of volunteers whose GBS colonization status is not known whereas ours is a comparison of GBS-colonized and noncolonized women and 2) their measure of phagocytosis is bactericidal index while ours was GBS engulfment. Therefore, the discrepancies in our work and Butko et al are easily explained by differences in study populations and experimental design.

The findings presented in this study and our previous work indicate that MBL may mediate phagocytic engulfment of GBS in colonized women and that this pathway could provide entry into an intracellular niche allowing GBS to evade clearance and establish persistent colonization.

ACKNOWLEDGEMENTS

Preliminary data from this study was presented in part at the American Society for Microbiology 101st General Meeting in Orlando, FL in May, 2001.

All participants gave informed consent according to the guidelines set by the Marshall University and University of Iowa Institutional Review Boards.

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APPENDIX B. ADDITIONAL FIGURES FOR CHAPTER 3

Figure 1. Mannose binding lectin exon-1 RFLP patterns. A 119bp segment of exon-1 of the MBL gene was amplified by PCR and digested by *Mbo*II, *Ban*I, or *Mlu*I restriction endonucleases. Allele type was determined by RFLP pattern. (Top) The wild-type allele was indicated by an uncut 119bp band in the *Mbo*II and *Mlu*I lanes and an 84bp band in the *Ban*I lane. This panel is representative of an individual homozygous for the wild-type allele. (Middle) The B allele was indicated by elimination of the *Ban*I restriction endonuclease site and the presence of the 119bp band in the *Ban*I lane. This panel is representative of an individual heterozygous for the B allele. (Bottom) The D allele was indicated by the insertion of an *Mlu*I restriction endonuclease site and the presence of a 95bp band in the *Mlu*I lane. This panel is representative of an individual heterozygous for the D allele. The C allele would have been indicated by the insertion of an *Mbo*II restriction endonuclease site and would have demonstrated a 79bp band in the *Mbo*II lane, however a C allele was not detected in any of the women in this study.

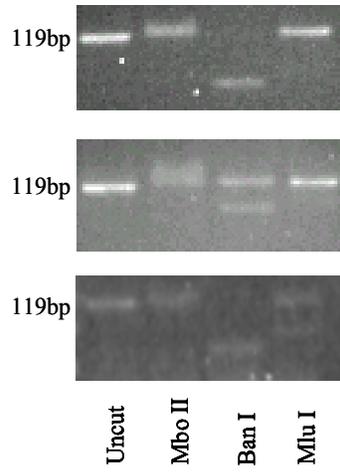
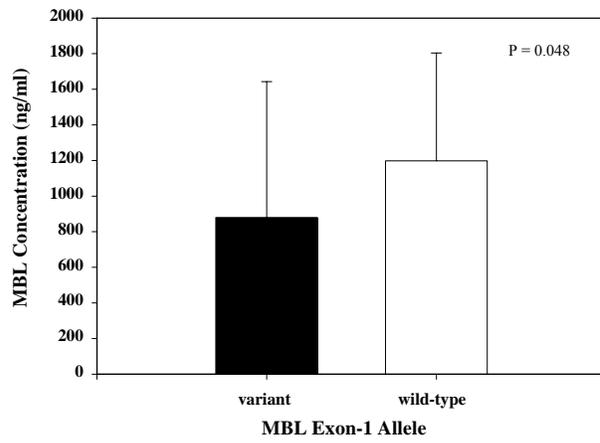


Figure 2. MBL exon-1 allele type and serum MBL concentration. MBL concentration was significantly higher in individuals with the wild-type alleles (n = 21) than in individuals that were heterozygous for a variant allele (n = 9) by Wilcoxon Rank Sum test ($P = 0.048$).



CHAPTER 4. ENHANCEMENT OF GROUP B STREPTOCOCCUS GROWTH AND VIRULENCE BY INTERLEUKIN-6

A paper to be submitted to *Infection and Immunity*.

Jennifer M. Smith^{1,2} and Susan H. Jackman^{1,2}

¹Department of Microbiology, Immunology, & Molecular Genetics

²Department of Obstetrics & Gynecology

Marshall University Joan C. Edwards School of Medicine, Huntington, WV

This chapter addresses interactions between another component of the innate immune system, pro-inflammatory cytokines, and GBS. Multiple reports indicate that cytokines may have a profound effect on bacterial growth and virulence. Therefore, we examined the effects of three pro-inflammatory cytokines on GBS growth and virulence. These experiments addressed specific aims 4-7.

ABSTRACT

Sepsis due to group B streptococcus (GBS) is mediated by the overwhelming release of pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6. Incubation of GBS with these cytokines revealed that IL-1 β and IL-6 concentrations of 100 ng/ml significantly enhanced GBS growth. However only for IL-6 is this level within the physiologic range observed during sepsis. Specific binding of IL-6 to GBS was demonstrated in a flow cytometry-based assay using FITC-conjugated IL-6. In addition to evaluate the effects of IL-6 on growth, mRNA expression of three common virulence factors was examined by RT-PCR and real time PCR. Only expression of the C5a peptidase was increased in response to IL-6 exposure. RAP-PCR was then used to globally identify additional GBS genes upregulated in response to IL-6. One of four products with increased expression after IL-6 exposure, an ATP-binding protein (ATPase) of an ABC transporter, appeared to be part of the operon encoding the GroES

and GroEL proteins which have been implicated in the pathogenesis of other bacterial species. Real time PCR verified the upregulation of mRNA expression of the ATPase as well as that of the GroE genes. Identification of other genes altered in response to IL-6 will provide a better understanding of GBS pathogenesis as well as reveal potential targets for future therapeutics.

INTRODUCTION

Group B streptococcus (GBS) is the primary etiologic agent associated with life-threatening bacterial infections in the neonatal period. In the hours or days following birth, infants infected by vertical transmission from a mother ano-vaginally colonized with GBS present with pneumonia, meningitis, or sepsis. Neonates with GBS sepsis have elevated plasma levels of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) (6). Such increases in cytokine concentrations, particularly TNF- α and IL-6, are associated with high mortality rates in both infants (37) and adults (36) with sepsis.

The cytokine network supplies important cues to surrounding tissues that lead to a myriad of host cellular responses. Characterization of bacterial responses to the host's cytokine milieu is a newly emerging field. Recently, cytokines including interleukin-2, granulocyte-macrophage colony-stimulating factor, and interleukin-1 have been observed to enhance *E. coli* growth (7, 30), while IL-6 can stimulate growth of *P. aeruginosa* (26) and *M. avium* (8).

The effects that the sepsis-associated cytokine-enriched environment may have on GBS have yet to be explored. We hypothesized that the pro-inflammatory cytokines induced by GBS infection may enhance the bacteria's growth and virulence

characteristics. We found that IL-6 enhanced GBS growth at high, but physiologically relevant, concentrations; that IL-6 specifically binds GBS; and that IL-6 increases expression of virulence-associated genes.

MATERIALS AND METHODS

Bacteria. A serotype III GBS strain, designated 2407, was isolated from an asymptomatic, colonized woman during a routine gynecologic exam. GBS 2407 was inoculated into Todd Hewitt broth (THB) and incubated overnight in 5% CO₂ at 37°C. Equal volumes of overnight culture and sterile 24% glycerol in phosphate buffered saline (PBS) were stored at -70°C until needed.

Cell culture. The human hepatoma cell line HuH-7, generously provided by Dr. Stanley M. Lemon from The University of Texas Medical Branch at Galveston, was maintained in RPMI-1640 (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and 25 µg/ml M-Plasmocin (InvivoGen, San Diego, CA) at 37°C in 5% CO₂.

Incubation of GBS 2407 with cytokines. THB inoculated with GBS 2407 from frozen stock was incubated overnight in 5% CO₂ at 37°C. The cultures were centrifuged at 1400 × g for 10 minutes and washed twice with RPMI-1640 without phenol red (Life Technologies). Approximately 10⁶ CFU/ml GBS 2407 were added to RPMI-1640 without phenol red containing varying concentrations (0, 1, 10, 100 ng/ml) of recombinant human TNF-α, IL-1β, or IL-6 (R & D Systems, Minneapolis, MN) and incubated in 5% CO₂ at 37°C for 8 hours. GBS 2407 growth was measured by optical density at 600 nm each hour. All growth experiments were repeated three times.

Co-incubation of GBS 2407 with IL-1 β and IL-6 was performed as above with the exception that one cytokine concentration was held at 10 ng/ml while the other was varied (0, 1, 10, 100 ng/ml).

Binding of IL-6 to GBS 2407. Binding of GBS 2407 was detected by flow cytometry using the human interleukin 6 biotin conjugate Fluorokine kit (R & D Systems). Approximately 10^6 CFU/ml GBS 2407 were incubated with human IgG (Sigma, St. Louis, MO) at 1mg/ml/ 10^6 cells for 15 minutes in order to block potential Fc-mediated interactions. Biotinylated IL-6 was added to the blocked cells and incubated for 1 hour at 4°C. Following this incubation, avidin-FITC was added and the cells were incubated for 30 minutes at 4°C in the dark. After washing, the cells were suspended in RDF1 buffer included in the Fluorokine kit. IL-6 binding to GBS 2407 was detected using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) with CellQuest software and measured by an increase in fluorescence greater than that of GBS 2407 incubated with the negative control protein, biotinylated soybean trypsin inhibitor. Pre-treatment of the biotinylated IL-6 with an anti-human IL-6 blocking antibody prior to addition to the Fc-blocked GBS 2407 cells demonstrated that the interaction with GBS was specific for IL-6.

RNA isolation and cDNA synthesis. GBS 2407 total RNA was isolated using modifications of the Trizol (Life Technologies) protocol or the RNeasy Mini Kit (Qiagen, Valencia, CA). Bacteria were pelleted at $1400 \times g$ for 10 minutes, washed with DEPC-treated water (Ambion, Austin, TX), and snap-frozen in liquid nitrogen. The frozen GBS 2407 pellets were lysed in either Trizol reagent or RNeasy buffer RLT using the Mini-BeadBeater (BioSpec Products, Bartlesville, OK) and 0.1 mm zirconia-silica beads

(BioSpec Products) for 3 minutes at 5000 rpm. After the beads settled, the lysate was removed and RNA was extracted according to the manufacturer's instructions. All RNA samples were stored in DEPC-treated water at -70°C.

RNA was obtained from HuH-7 cell monolayers by lysing in the culture dish using Trizol reagent. Total RNA was isolated according to the manufacturer's instructions and stored in DEPC-treated water at -70°C.

One microgram of total RNA from either GBS 2407 or HuH-7 cells was reverse transcribed to cDNA in a standard reaction using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies) and random hexamer primers. Parallel samples were run without the addition of M-MLV reverse transcriptase to be used as a negative control in the PCR reactions.

PCR. Standard 50 µl PCR reactions using 1 µl cDNA, 0.4 µM GBS gene-specific primers and 2.5 U *AmpliTaq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) were conducted under the following conditions: 30 cycles of 95°C for 45 seconds, 53°C for 45 seconds, and 72°C for 1 minute, with a final 7 minute extension at 72°C. Sequences of GBS gene-specific primers designed using OLIGO 5.0 (National Biosciences, Hamel, MN) and synthesized by the Marshall University DNA Core Facility are listed in Table 1. PCR products were visualized on a 3% NuSieve-GTG and SeaKem-GTG agarose (BioWhittaker Molecular Applications, Rockland, ME) gel containing GelStar nucleic acid stain (BioWhittaker Molecular Applications). Gels were photographed with the Kodak Digital Science 1D Electrophoresis Documentation and Analysis System and band intensities were measured with the system's associated software (Eastman Kodak

Table 1. GBS-specific primer sequences.

Primer name	Sequence	Source (Ref.)
16S forward	5'GCGGCTCTCTGGTCTGTAAC3'	Genbank Z22808 (32)
16S reverse	5'TAAGGTTCCTTCGCGTTGCTT3'	Genbank Z22808 (32)
<i>cpsE</i> forward	5'GCAGAAGCGACGCCTTAGTT3'	Genbank AF163833 (3)
<i>cpsE</i> reverse	5'GCTCCTGTCCCGAGTAAAAC3'	Genbank AF163833 (3)
<i>scpB</i> forward	5'GCCAATAGCAGCAAACAAGT3'	Genbank U56908 (5)
<i>scpB</i> reverse	5'GCCAAAATCACCTCGGAAAC3'	Genbank U56908 (5)
<i>cyl</i> forward	5'ATAGCCTTTCTTTTCCACTG3'	Genbank AF157015(31)
<i>cyl</i> reverse	5'TGCCATAATCCTTCTCTTCT3'	Genbank AF157015(31)
<i>atpase</i> forward	5'CAGGGGACAATCACAT3'	RAP-PCR sequence
<i>atpase</i> reverse	5'GCAGCATCAGTCTTCAT3'	RAP-PCR sequence
<i>groEL</i> forward	5'GCCTGGATTTGGTGAT3'	RAP-PCR sequence
<i>groEL</i> reverse	5'AGTTTCAGTGCCGCTAC3'	RAP-PCR sequence
<i>groES</i> forward	5'TGTCGGTGGTTTTGTTC3'	RAP-PCR sequence
<i>groES</i> reverse	5'ACCAGCACCATTTTCG3'	RAP-PCR sequence

Company). GBS *cpsE*, *scpB*, and *cyl* PCR products were normalized against a 16S product.

GBS Protein Isolation. GBS 2407 protein pellets snap-frozen in liquid nitrogen were disrupted in protein lysis buffer (0.1 M Tris, pH 7.5; 0.5 M EDTA; glycerol; leupeptin (1 mg/ml); aprotinin (1 mg/ml); pepstatin (1 mg/ml); PMSF (17.4 mg/ml); PBS) using the Mini-BeadBeater and 0.1 mm zirconia-silica beads for 3 minutes at 5000 rpm. The GBS lysates were centrifuged at 14,000 x g for 10 minutes at 4°C to pellet cellular debris. The supernatant was concentrated using Centricon YM-3 centrifugal filter devices (Millipore, Bedford, MA) according to the manufacturer's instructions. The protein concentration was measured using the BCA Protein Assay kit from Pierce (Rockford, IL).

Western Blot Analysis. Eight micrograms of concentrated GBS protein lysate were loaded onto a denaturing 4 –15% linear gradient Criterion polyacrylamide gel (BioRad, Hercules, CA). The proteins were transferred to a 0.45 µm nitrocellulose membrane (Stratagene, Cedar Creek, TX) by semi-dry electroblotting. Membranes were blocked overnight at 4°C with Tris-buffered saline – 0.1% Tween 20 (TBS-T) plus 3% dry nonfat milk. Blots were incubated with a 1:750 dilution of anti-GroEL polyclonal antibody (StressGen Biotechnologies, Corp., Victoria, BC Canada) in TBS-T-milk for 1 hour at room temperature, followed by 3 washes in TBS-T. Incubation with a 1:20,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (BioRad, Hercules, CA) was followed by chemiluminescent detection using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce).

Real Time PCR Standards. GBS 2407 virulence gene PCR products were cloned into the pCR4.0-TOPO vector and transformed into TOP10 One Shot *E. coli* using the TOPO-

TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Ampicillin-resistant transformants were PCR-screened for the appropriate insert. An insert-positive colony was then inoculated into LB broth with 50 µg/ml ampicillin and incubated with shaking for 12-16 hours at 37°C. Plasmid DNA isolated using the Qiagen Plasmid Mini Kit was used as template in PCR reactions as described above. The PCR products were separated on a 2% NuSieve GTG – SeaKem GTG agarose gel containing GelStar nucleic acid stain and then extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). The molecules of standard per microliter were calculated for the extracted DNA and serial ten-fold dilutions ranging from 10^8 to 10^1 molecules/µl were made in nuclease-free water (Promega Corporation). The standard dilutions were frozen at -20°C until use.

Real Time PCR. To quantify GBS 2407 virulence gene expression real time PCR was performed using the iCycler Real Time PCR System (BioRad). All reactions included 1 µl standard DNA dilution or GBS cDNA in addition to standard reaction components and 3 µl of a 1:50,000 dilution of SYBR Green I nucleic acid stain (BioWhittaker Molecular Applications). iCycler conditions were as described for the PCR reactions minus the final 7 minute extension. All standards were run in duplicate reactions and all experimental samples were run in triplicate. Experimental sample mRNA copy number was calculated from a standard curve using the iCycler Optical System Interface software version 2.3 (BioRad).

Haptoglobin real time PCR standards were prepared as described above for GBS standards and reactions were conducted using haptoglobin gene-specific primers and cycling conditions previously described by Piniero (29).

Random Arbitrarily Primed-PCR (RAP-PCR). Two microliters of GBS cDNA were added to PCR reactions and amplified using the Advantage cDNA Polymerase Mix (Clontech, Palo Alto, CA) and pairs of random primers from the Delta Differential Display Kit (Clontech). Cycling conditions were 94°C for 1 minute; 40 cycles of 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds; followed by a final extension at 72°C for 10 minutes. RAP-PCR products were visualized on a 2% Metaphor agarose (BioWhittaker Molecular Applications) containing GelStar nucleic acid stain in a horizontal gel electrophoresis apparatus for 4 hours at 80 volts. A 100 bp step ladder (BioWhittaker Molecular Applications) was used to size RAP-PCR products. Differential expression of RAP-PCR products was verified by real time PCR as described above using primers from Table 1.

Identification of Differentially Expressed Products. Differentially expressed bands were extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen) and reamplified using the same random primer set. Plasmid DNA isolated from TOP10 One Shot *E. coli* transformed with pCR4.0 TOPO containing the PCR product was sequenced by the Marshall University DNA Core Facility. DNA sequence obtained from the differentially expressed RAP-PCR products was compared to known bacterial genes and genomes using the Basic Local Alignment Search Tool (1) (BLASTN and BLASTX algorithms) provided on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/BLAST/). The RAP-PCR sequence was then submitted to Dr. Philippe Glaser and Dr. Frank Kunst at the Laboratoire de Genomique des Microorganismes Pathogenes at the Institut Pasteur (Paris, France) to obtain the complete gene sequence and surrounding sequences.

Statistics. All statistical analyses were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA). Two-way analysis of variance was used for comparisons of GBS growth measurements. Student's *t*-test was used for comparisons of gene expression. Differences were considered statistically significant when $P < 0.05$.

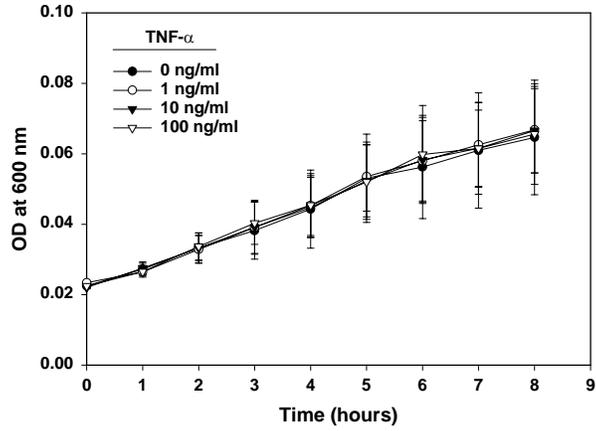
RESULTS

Cytokine effects on GBS growth. To determine the effects of proinflammatory cytokines on its growth, GBS 2407 was incubated with concentrations of TNF- α , IL-1 β , or IL-6 ranging from 0-100 ng/ml for 8 hours. Optical density readings at 600 nm revealed that GBS growth was significantly increased by exposure to 100 ng/ml IL-1 β or IL-6 ($P < 0.05$), while TNF- α had no effect at any concentration (Fig. 1). Because this concentration of IL-1 β was supraphysiologic, even for a septic state, potential synergistic effects between IL-1 β and IL-6 were evaluated. Figure 2 shows the results of incubation of GBS with one cytokine concentration held at 10 ng/ml and the other varied from 0-100 ng/ml. An increase in GBS growth was only observed when the concentration of the second cytokine was 100 ng/ml. This growth enhancement was not different from what was observed for the individual cytokine treatments (Fig. 1) indicating that there was no synergism between IL-1 β and IL-6. These results suggested that only IL-6 is likely to enhance GBS growth *in vivo*. Consequently, only the effects of IL-6 exposure on GBS growth were further investigated.

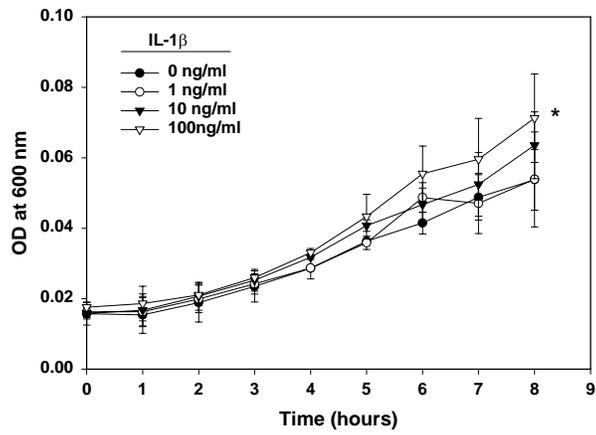
A concern arose that perhaps this increase in GBS growth following IL-6 exposure was nonspecific and due to the bacteria's use of IL-6 as a nutrient source. To rule out this possibility the requirement for IL-6 biologic activity in enhancement of GBS

FIG. 1. IL-1 β and IL-6, but not TNF- α , increase GBS growth. GBS were incubated with increasing concentrations of TNF- α (A), IL-1 β (B), and IL-6 (C) at 37°C in 5% CO₂. GBS growth was monitored by optical density at 600 nm every hour for 8 hours. Two-way analysis of variance indicated a significant increase in GBS growth at 100 ng/ml IL-1 β or IL-6 ($P < 0.05$) versus growth with no cytokine.

A



B



C

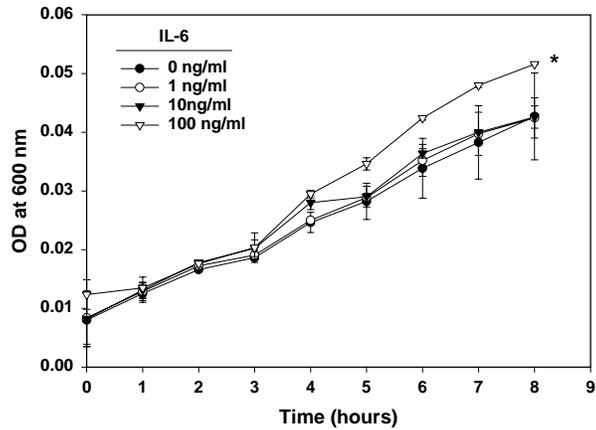
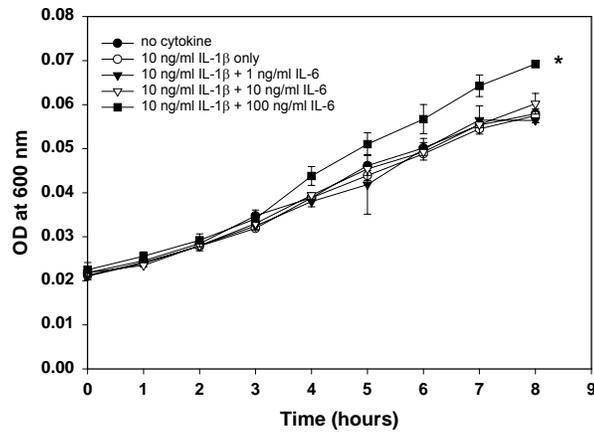
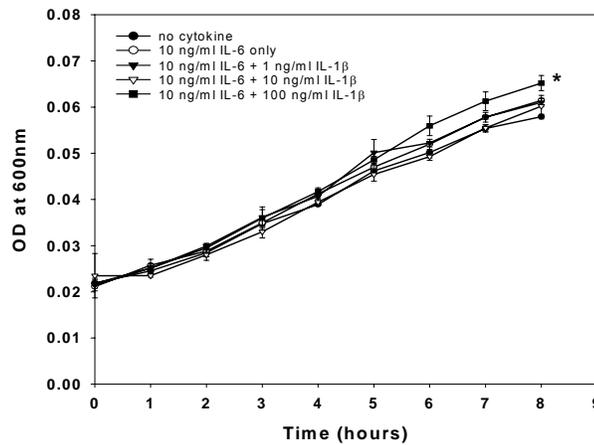


FIG. 2. IL-1 β and IL-6 do not act synergistically to enhance GBS growth. GBS were incubated with IL-1 β at 10 ng/ml and varying concentrations of IL-6 (A) or with IL-6 at 10 ng/ml and varying concentrations of IL-1 β (B). GBS growth was monitored by optical density at 600 nm every hour for 8 hours. Two-way analysis of variance indicated significant growth enhancement only occurred at IL-1 β and IL-6 concentrations of 100 ng/ml ($P < 0.05$).

A



B



growth was tested. Inactivation of IL-6 was accomplished by boiling for 20 minutes. To verify inactivation of IL-6 biologic activity, confluent monolayers of HuH-7 liver carcinoma cells were incubated with 0 ng/ml, 100 ng/ml, or 100 ng/ml inactivated IL-6 for 24 hours in 5% CO₂ at 37°C. Real time PCR analysis was performed to examine the IL-6-induced expression of haptoglobin, an acute phase reactant protein. The expression of the haptoglobin gene was significantly decreased by the inactivation of IL-6 ($P < 0.001$) compared to expression induced by the untreated IL-6 (See Appendix C, Figure 1). When inactivated IL-6 was supplied in the media, the stimulation of GBS growth was abolished indicating that IL-6 biologic activity is required for its enhancement of GBS growth (Fig. 3). The necessity for biologically active IL-6 in the augmentation of GBS proliferation implies that IL-6 functions as more than a nutritional source for GBS and may influence additional bacterial characteristics including virulence properties.

IL-6 binding to GBS. Cytokines mediate their effects by binding receptors on the host cell surface. We investigated IL-6 binding to GBS using flow cytometry. An increase in bacteria-associated fluorescence after incubation of GBS with FITC-conjugated IL-6 indicated an interaction between GBS and IL-6 (Fig. 4). Treatment of the FITC-IL-6 with an anti-human IL-6 blocking monoclonal antibody prior to the incubation with GBS decreased the fluorescence to the same level as the negative control thereby illustrating the “specificity” of the interaction.

FIG. 3. IL-6 biologic activity is required for enhancement of GBS growth. GBS were incubated with 0 ng/ml, 100 ng/ml active, or 100 ng/ml inactivated IL-6. GBS growth was monitored by optical density at 600 nm for 8 hours. Two-way analysis of variance indicated that treatment with inactivated IL-6 was not significantly different from no IL-6 treatment. Asterisk indicates significant difference ($P < 0.05$) from both no IL-6 and inactivated IL-6 treatment.

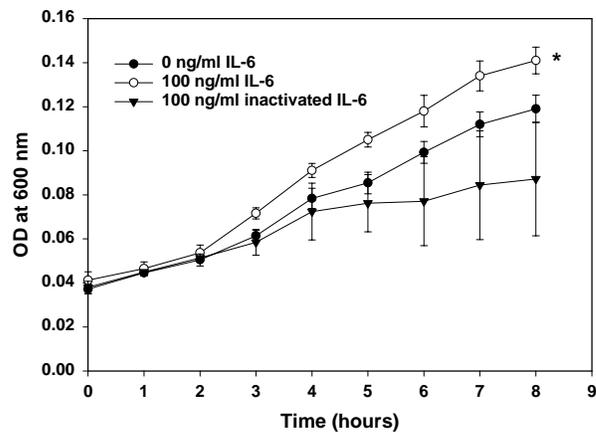
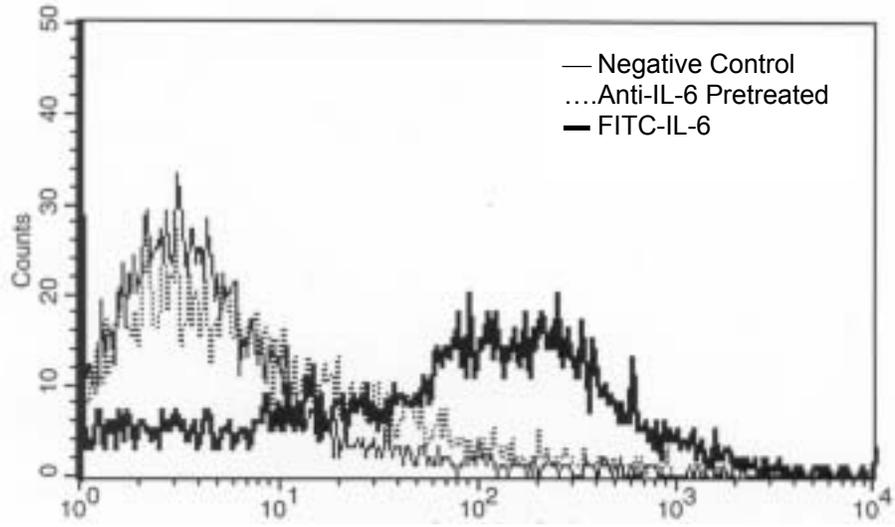


FIG. 4. GBS binds IL-6. GBS incubated with FITC-conjugated IL-6 showed an increase in fluorescence indicating binding of IL-6 to the bacteria. GBS incubated with FITC-IL-6 pretreated with an anti-human IL-6 blocking antibody exhibited a fluorescence level equivalent to that of the negative control indicating that binding was specific for IL-6.



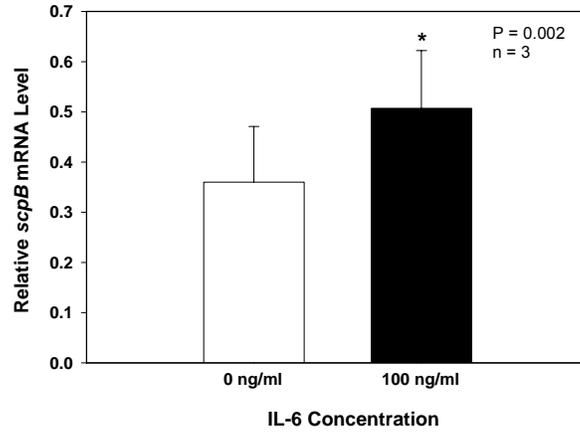
IL-6 effects on expression of GBS virulence factors. Ross et al (33) report that growth rates alter expression of several GBS virulence factor genes. Because IL-6 increased GBS growth, RT-PCR and real time PCR analyses of the effects of IL-6 exposure on the mRNA expression of three GBS virulence factors (cytolysin, capsule polysaccharide, and C5a peptidase) were performed. No difference in expression of the GBS cytolysin gene, *cyl*, or of a capsule polysaccharide gene, *cpsE*, was observed in IL-6-treated GBS compared to nontreated bacteria (See Appendix C, Figure 2). Expression of *scpB*, the GBS C5a peptidase gene, was significantly increased after IL-6 exposure by RT-PCR analysis (Fig. 5). These data indicate that elevated IL-6 concentrations may boost GBS's virulence.

RAP-PCR. A more global investigation of the alteration of GBS gene expression following IL-6 treatment was then conducted using RAP-PCR, a modification of differential display PCR. Four products showed increased expression upon IL-6 treatment. Results from DNA sequencing and BLAST comparisons revealed that the products were homologous to genes encoding an ATP-binding protein (ATPase) of an ABC transporter (Fig. 6), the DNA-dependent RNA polymerase β' subunit, an unknown protein, the 23S rRNA subunit (data not shown). Of these four products the most intriguing prospect for additional investigation was the ATP-binding protein because of the important roles that ABC transporters play in bacteria, which range from import and export of nutrients and wastes to antibiotic resistance to secretion of virulence factors.

In order to gain insight into possible functions for this ATPase and its associated transporter, nucleotide sequence for the regions flanking the sequence represented by the

FIG. 5. Expression of the GBS C5a peptidase gene (*scpB*) may be increased by IL-6 exposure. Total RNA was isolated from GBS incubated with or without 100 ng/ml IL-6. Expression of *scpB* was analyzed by RT-PCR (A) and real time PCR (B). IL-6 exposure increased *scpB* expression ($P < 0.05$ by Student's *t*-test) by RT-PCR. The difference was not statistically significant by real time PCR analysis.

A



B

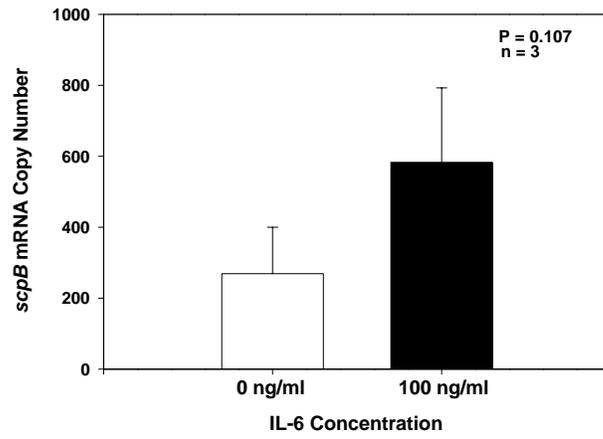
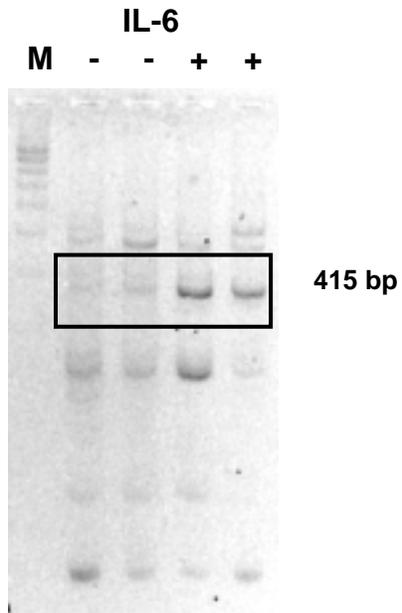


FIG. 6. RAP-PCR identified a 415 bp product that is upregulated upon GBS exposure to 100 ng/ml IL-6 for 8 hours. Lane 1 (M) is a 100 bp ladder used to estimate RAP-PCR product size. Lanes 2 and 3 (-) represent GBS not exposed to IL-6, while lanes 4 and 5 (+) represent GBS exposed to IL-6.



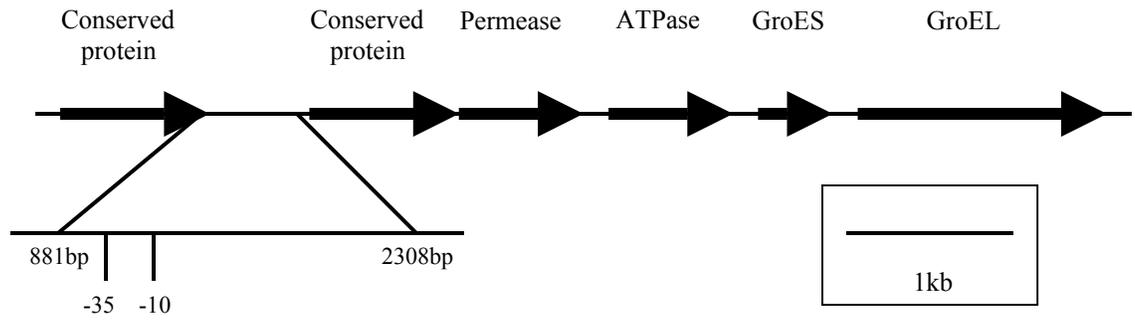
ATPase RAP-PCR product was obtained from the databases for the *Streptococcus agalactiae* genome sequencing project (courtesy of Drs. Philippe Glaser and Frank Kunst at the Institut Pasteur). A schematic of the 6.5 kilobases of DNA sequence surrounding the ATPase gene is shown in Figure 7A. Directly upstream of the gene encoding the ATP-binding protein are genes coding for two conserved proteins with unknown function and a gene for an ABC transport permease. Downstream of the ATPase gene are the genes of the GroE operon.

Real time PCR analysis of expression of the ATPase, GroES, and GroEL genes was conducted (Fig. 7B, C, D). Expression of all three genes was significantly enhanced in response to IL-6 ($P < 0.05$). Interestingly, the increase was approximately six-fold for each when compared to that of untreated GBS.

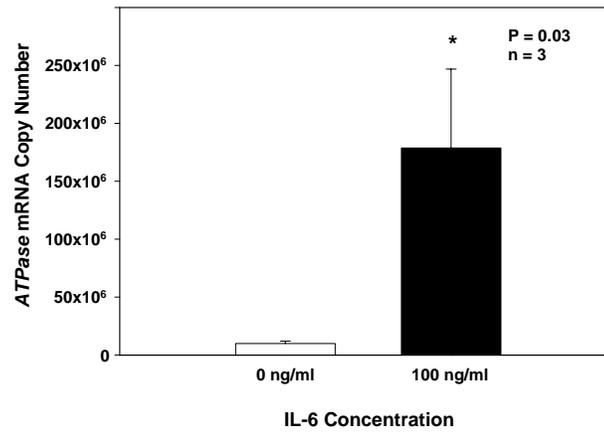
Due to the similar pattern of gene expression upon IL-6 treatment, as well as the proximity of the genes on the GBS chromosome, the possibility that the ATPase and GroE genes form a single operon was examined. Analysis of the DNA sequence upstream of the predicted ABC transporter, between bases 880 and 1367, using Omega 2.0 software (Accelrys, San Diego, CA) yielded only one reasonable candidate for promoter sites (-35 site at bases 988-993 and -10 site at bases 1011-1016). Further evidence that the genes of the ABC transporter and the GroE operon may constitute one operon was that a single transcript incorporating the ATPase through GroEL was identified by RT-PCR (Fig. 8) and verified by DNA sequencing. These data strongly suggest that the ATPase and the GroE genes form a functional genetic unit.

FIG. 7. DNA sequence and expression analysis of genes surrounding the ATPase gene. (A) Schematic of DNA sequence surrounding the ATPase identified by RAP-PCR. The arrows indicate the directionality of each gene. The expanded area represents the region spanning bases 881 to 2308 of the 6.5kb DNA sequence segment. This expanded region contains the -35 and -10 promoter sites (at bases 988-993 and 1011-1016, respectively) for the transcript encoded by the downstream genes. (B-D) ATPase, GroES, and GroEL mRNA expression are increased in IL-6-treated GBS. Total RNA isolated from GBS treated with no IL-6 or 100ng/ml IL-6 was analyzed by real time PCR for expression of ATPase (B), *groES* (C), and *groEL* (D). All three genes demonstrated a significant increase in expression following IL-6 exposure for 8 hours ($P < 0.05$). (E) Western blot analysis of GBS GroEL protein expression after IL-6 treatment for 10 hours. Lanes 1 and 2 represent the untreated GBS from two separate experiments, lanes 3 and 4 represent the matching IL-6-treated GBS from the same experiments, and lane 5 is 10 ng of a positive control GroEL protein. An increase in GroEL protein expression is seen when comparing the IL-6-treated GBS to their respective untreated controls (lane 3 versus lane 1 and lane 4 versus lane 2).

A



B



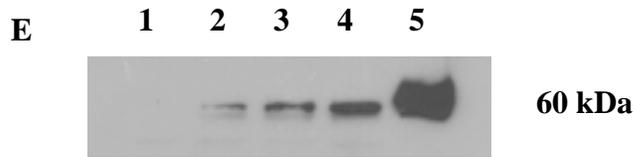
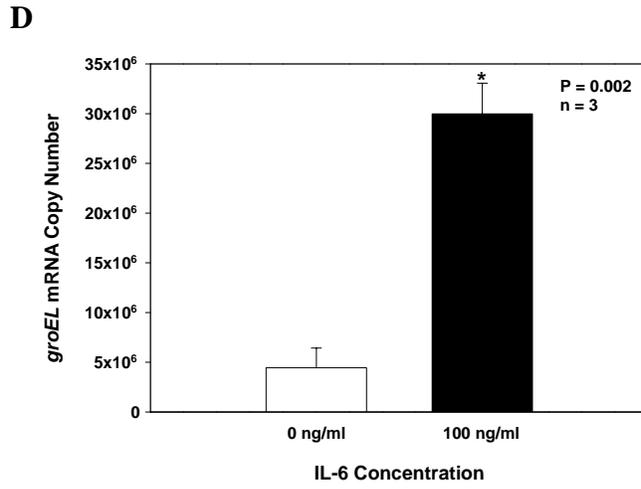
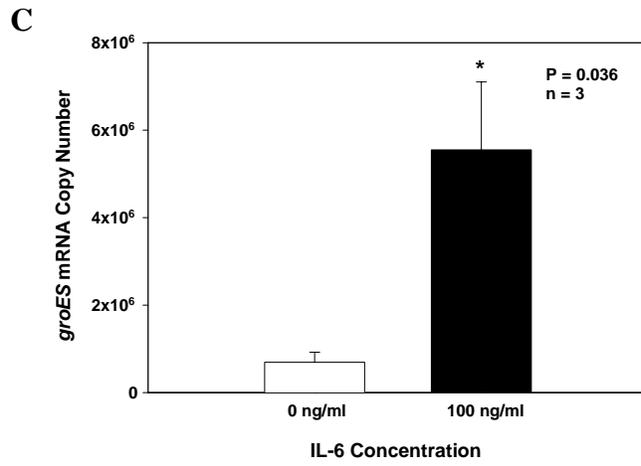
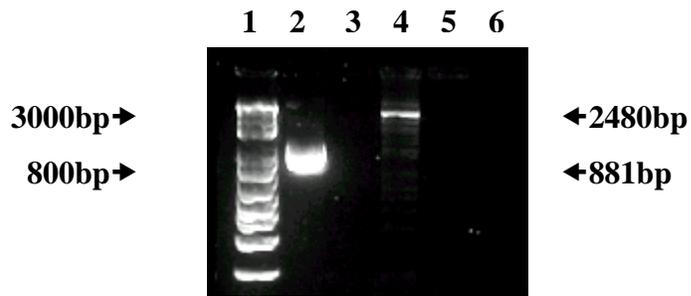


FIG. 8. RT-PCR analysis indicates that the genes encoding the ATPase, GroES, and GroEL are transcribed as a single message. Lane 2 is a transcript spanning the ATPase gene through the GroES gene and lane 3 is the corresponding negative control. Lane 4 is a transcript spanning the ATPase gene through the GroEL gene and lane 5 is the corresponding negative control. Lane 6 is a water control. Lane 1 is a 1Kb Plus DNA Ladder (Invitrogen) which was used to verify the predicted sizes of the PCR products, 881 bp for ATPase-GroES and 2480 bp for ATPase-GroEL.



Western blot analysis. To confirm that the increase in GroEL mRNA expression leads to an increase in the protein levels, total protein lysates from IL-6 treated and nontreated GBS were separated by denaturing polyacrylamide gel electrophoresis and GroEL protein was detected by an anti-GroEL monoclonal antibody. Although GroEL mRNA from GBS treated for 8 hours with IL-6 was increased, analysis of protein at this time period did not show any difference in expression from nontreated GBS (See Appendix C, Figure 3). However, GBS treated for 10 hours with IL-6 showed an increase in GroEL expression compared to untreated GBS (Fig. 6E).

DISCUSSION

Environmental conditions greatly influence cellular/organismal responses. During the initiation of an infection or during colonization, factors produced by both the host and the microbe direct the outcome. When the host's immune system encounters potential pathogens, a multitude of cytokines are secreted to instruct the host's defenses on how to eliminate the threat. Little is known about how this cytokine-enriched microenvironment may directly affect the microorganism that prompted the immune response. For this reason, we chose to examine the effects that pro-inflammatory cytokines generated in response to group B streptococcus have on the bacteria's growth and expression of virulence.

The predominant cytokines secreted during GBS infection and sepsis are TNF- α , IL-1 β , and IL-6 (2, 22, 39). Incubation of a serotype III GBS isolate with increasing concentrations of these three cytokines revealed that high concentrations of IL-1 β and IL-6 significantly enhanced bacterial proliferation (Fig. 1), but only the IL-6 concentration was within physiologic range. In addition, the increase in GBS growth in

response to IL-6 exposure required IL-6 biologic activity (Fig. 3). Our data is not unlike a number of other studies demonstrating that cytokines possess bacterial growth promoting activity. These studies reveal that growth of virulent *E. coli* is increased by exposure to interleukin-2, granulocytes-macrophage colony-stimulating factor, interleukin-1, and interferon- γ (7, 30), while *M. avium* growth is enhanced by exposure to interleukin-6 and interleukin-1 α (8). Likewise, others report that *M. tuberculosis* growth is augmented by transforming growth factor- β (18); *L. monocytogenes* by colony-stimulating factor-1, and interleukin-3 (9); and *S. aureus* by tumor necrosis factor- α , interleukin-1 β , and interleukin-6 (26).

Similar to data presented in the current study, Denis et al (7) observe that heat-inactivation of interleukin-2 prevents its growth promotion of virulent *E. coli*. Luo et al (24) report that binding of tumor necrosis factor- α to *S. flexneri* requires the cytokine's biologic activity. The necessity for cytokine biologic activity suggests that cytokines may exert their effects on the bacteria in ways that may in fact alter the bacteria's virulence characteristics.

In the host, cytokines bind specific receptors in order to induce their effects. Our data showed that IL-6 does in fact bind to GBS and that this binding can be blocked by an anti-IL-6 monoclonal antibody (Fig. 4A). In other studies, binding interactions between TNF- α and *S. flexneri*, *S. typhimurium*, and *E. coli* (24); IL-1 β and *S. aureus* (20) and *Y. pestis* (42); and interleukin-2 and *C. albicans* (38) have been observed. Binding of cytokines to the bacterial surface again suggests that the cytokines may be responsible for influencing the bacteria beyond the enhancement of bacterial proliferation. Perhaps, IL-6 is modulating GBS pathogenicity by binding a bacterial surface molecule that is

associated with a two-component signal transduction system that initiates a phosphorelay cascade involved in the activation of virulence expression (14, 19).

Because alterations in growth rates and cell densities (21, 40, 4, 25) are often associated with changes in bacterial virulence characteristics, we initially chose three of the most well-characterized GBS virulence factors (35) and analyzed their mRNA expression in response to IL-6 exposure. Of these, only the C5a peptidase gene expression appeared to be upregulated by IL-6 exposure. However, these results suggested that other virulence factors or proteins may in fact be candidates for study.

To this end, RAP-PCR analysis of GBS gene expression in response to IL-6 treatment was conducted. The results indicated that expression of an ATPase of an ABC transporter upstream of the GBS GroE heat shock proteins was significantly increased (Fig. 6A). Real time PCR confirmed these data, as well as demonstrated that expression of GroEL and GroES were also concomitantly increased (Fig. 6B, C, D). Western blot analysis also showed an increase in GroEL protein levels in IL-6-treated GBS (Fig. 6E).

In addition to its association with the heat shock response, expression of GroEL has more recently been correlated with pathogenesis in many bacterial species. For example, GroEL shows increased expression upon contact with host cells in *L. pneumophila* (13), *L. monocytogenes* (12), and *M. tuberculosis* (27). The GroEL protein also appears to become surface-exposed and mediates attachment and/or internalization to the host cells during infection with *L. pneumophila* (13), *H. influenzae* (16), *S. typhimurium* (10), *H. pylori* (28), *C. difficile* (17), and *H. ducreyi* (11). In order for these bacteria to use GroEL for attachment, the protein must translocate from the cytoplasm to the bacterial cell surface. In *C. difficile* surface expression has been proposed to be via a

type III secretion system or an ABC transporter (17), although no empirical evidence has yet been reported. Genomic sequence analysis of the region surrounding the GBS ATPase gene identified a single promoter region between the two conserved proteins upstream of the ATPase gene. As no other potential promoter sites were recognized within the sequence, it appeared that the ABC transporter containing the ATPase gene and the GroE genes were part of one transcriptional unit. RT-PCR analysis revealed a single mRNA transcript spanning the ATPase gene through the GroEL gene. Detection of the GroE genes and the ABC transporter as one message suggests that GroEL may become surface-exposed via an ABC transporter in GBS.

In summary, our data provide evidence that GBS exposure to high concentrations of IL-6 may also induce a stress-associated response in the bacteria in reaction to an environment that mimics an environment similar to that which would be encountered during GBS neonatal sepsis. Based on results from this study we speculate that in infants with GBS disease that the GBS GroEL protein may be upregulated and become surface-expressed. Supporting this hypothesis, patients suffering from infections with *B. pseudomallei* (41), *L. kirschneri* (15), *C. trachomatis* (34), and *S. pyogenes* (23) have high antibody titers to the GroEL protein from the infecting bacterial species.

Current strategies for prevention of GBS disease center around the design of a vaccine using immunogenic GBS surface proteins in conjunction with the serotype-specific capsular polysaccharides. Identification of factors upregulated in response to cytokine exposure, or to an environment similar to that encountered in the host, may uncover GBS proteins that could be included as conjugates in a GBS vaccine to boost the vaccine's efficacy.

ACKNOWLEDGEMENTS

Funding for this work was provided in part by a Sigma Xi Grants-in-Aid-of-Research awarded to J. Smith.

We thank Drs. Philippe Glaser and Frank Kunst at the Laboratoire de Genomique des Microorganismes Pathogenes at the Institut Pasteur, Paris, France, for providing the genomic DNA sequence surrounding the ATPase gene. We also thank Dr. Stanley Lemon from the University of Texas Medical Branch at Galveston for providing us with the HuH-7 cell line.

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APPENDIX C. ADDITIONAL FIGURES FOR CHAPTER 4

FIG. 1. Inactivation of IL-6 abolishes the IL-6-inducible expression of the haptoglobin gene by HuH-7 cells. GBS was incubated with no IL-6, 100 ng/ml IL-6, or 100 ng/ml inactivated IL-6 for 8 hours at 37°C. Asterisk indicates that haptoglobin mRNA expression at 100 ng/ml IL-6 is significantly different from that at 0 ng/ml IL-6 and 100 ng/ml inactivated IL-6 by Student's *t*-test.

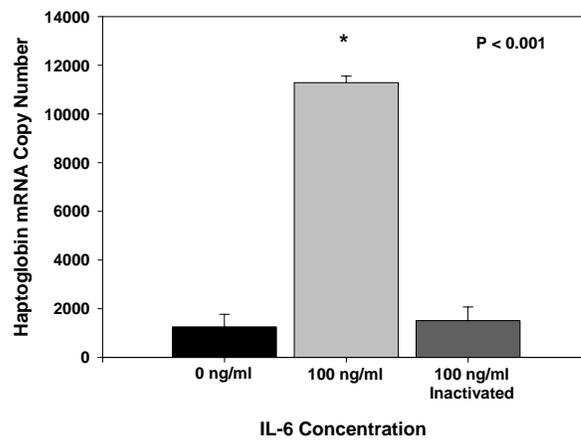
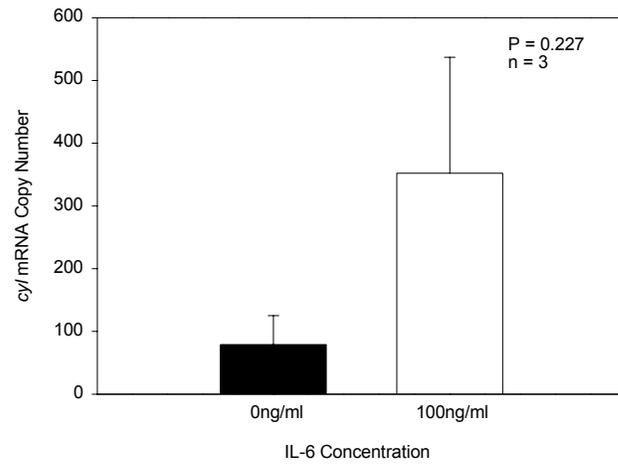
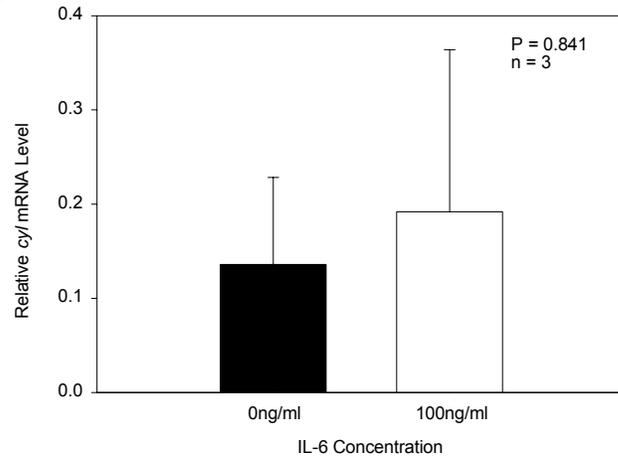


FIG. 2. mRNA expression analysis of two GBS virulence factor genes, *cyl* and *cpsE*, does not show an increase following IL-6 exposure. (A) Expression of the GBS cytolysin/hemolysin gene (*cyl*) is not increased by IL-6 exposure using RT-PCR (top) and real time PCR (bottom). (B) Expression of the GBS capsule polysaccharide gene E (*cpsE*) is not increased by IL-6 exposure using RT-PCR (top) and real time PCR (bottom).

A



B

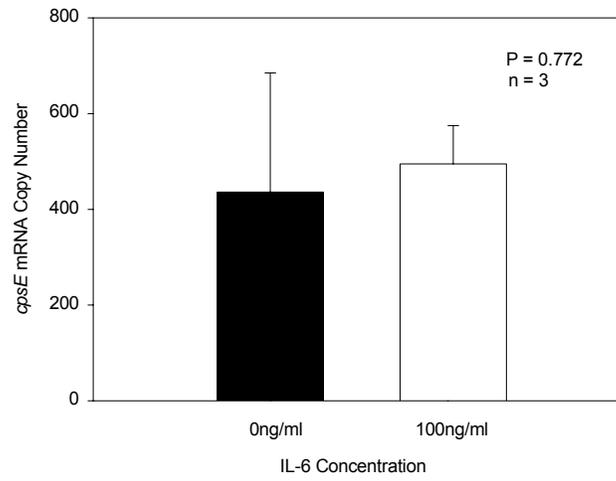
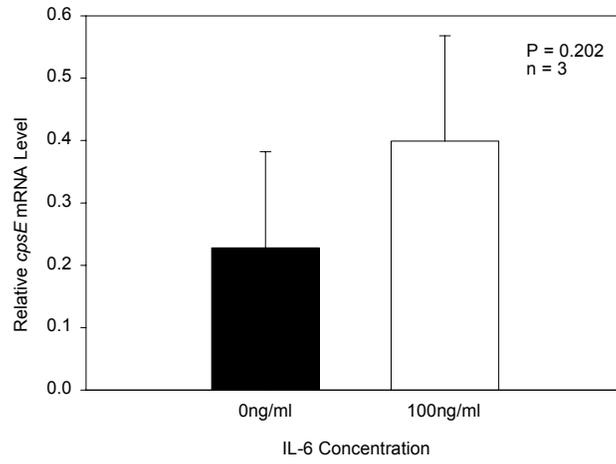
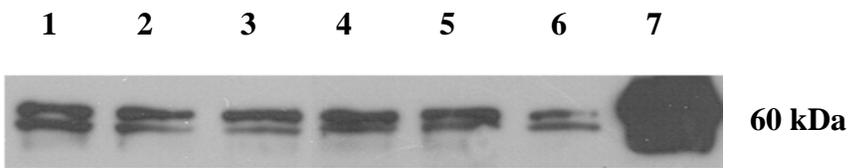


FIG. 3. The GBS GroEL protein does not show an increase in expression by densitometric analysis after 8 hours of IL-6 exposure. Lanes 1-3 represent untreated GBS from 3 separate experiments. Lanes 4-6 represent IL-6-treated GBS from the corresponding experiments. Lane 7 is 10 ng of a positive control GroEL protein.



CHAPTER 5: GENERAL DISCUSSION

Group B streptococcus has emerged over the last 30 years as one of the primary threats to infants during the first days and months of their lives. Thousands of neonates are put at risk each year because their mothers carry this potential life-threatening bacterium in the gastrointestinal and/or urogenital tract. Not only is GBS a major health concern for obstetricians and pediatricians, but it can also place a significant financial burden on the families of infected infants as well as the treatment facility. Since the mid-1990's emphasis on identifying women at risk, prophylactic treatment during pregnancy, and design of a GBS vaccine has been the principal focus of both clinicians and researchers alike. Although some research has studied immunity to GBS, knowledge concerning the interactions of the human immune system with GBS remains limited. Understanding these interactions would increase the likelihood of designing successful therapeutic and preventative strategies.

During the time when a microorganism is trying to establish colonization or an infection and the immune system is trying to thwart the microbe's attempts, many complex interactions take place. For a number of bacterial species, the fight for survival involves evading destruction by using the host immune response to its own advantage. For example, phagocytosis of bacteria that survive intracellularly within macrophages can lead to the creation of a protective niche for the microbe within the immune system itself.

We speculated that, like many other bacterial species, interactions between GBS and the human immune system may contribute to colonization by GBS, or at least do not result in the appropriate clearance of GBS. As phagocytosis by cells of the immune

system is one of the earliest steps in host defense, we examined engulfment of GBS and the respiratory burst activity subsequent to engulfment in phagocytes from GBS-colonized and noncolonized women. We observed that monocytes from pregnant, GBS-colonized women engulfed significantly greater numbers of GBS, while expelling the superoxide (generated to kill the engulfed GBS) into the extracellular environment. This would seem to support the notion that GBS is capable of turning the host's immune response to its own advantage. Internalization of GBS into the monocytes, in which the antimicrobial defenses have been undermined, may create a protective niche for the bacteria. Further support that GBS may become sequestered within the monocyte/macrophage as a haven for GBS comes from Valentin-Weigand et al (1996) and Cornacchione et al (1998) whose work provides evidence that GBS survive intracellularly for extended periods of time.

Phagocytosis of bacteria is enhanced by opsonization with bacteria-specific antibodies, complement components, or lectins. Numerous studies have demonstrated that the most effective opsonins for enhancing GBS clearance are antibodies to the serotype-specific capsule polysaccharide (Baker et al, 1976; Shigeoka et al, 1978; Kallman et al, 1998; Campbell et al, 2000) and components of the classical complement pathway (Shigeoka et al, 1978). However, most individuals fail to produce sufficient quantities of anti-capsular antibodies after natural infection (Baker et al, 1976) or vaccination (Baker et al, 1988). Even in the absence of antibody opsonization and activation of the classical complement pathway, the alternative pathway should be activated but is not in response to GBS because of bacteria's high sialic acid content

capsule. Little is known regarding the interaction between GBS and the final opsonic pathway for enhancement of phagocytosis, the lectin pathway.

We originally hypothesized that GBS colonization was associated with a deficiency in one of the lectin pathway components, mannose binding lectin. Initial data (Smith et al, 2001) demonstrating that a greater number of GBS-colonized women carried variant alleles for the first exon of the MBL gene, which have been associated with serum MBL deficiencies, supported this hypothesis. However, with the addition of more subjects this association disappeared. Additionally, no association between GBS colonization status and polymorphisms in the MBL gene promoter was observed. We anticipated that measurement of MBL concentrations from sera of GBS-colonized and noncolonized women would reflect the genetic findings. Interestingly, the GBS-colonized women had significantly higher levels of MBL in their sera compared to noncolonized women.

Based on the results of the MBL serum ELISAs, we realized that perhaps our initial hypothesis concerning GBS colonization association with MBL deficiency was the opposite of what was really occurring. We focused our efforts on investigating the possibility that MBL may function in mediating phagocytosis of GBS in the absence of specific antibody. To evaluate this hypothesis, we conducted additional phagocytic engulfment assays using serum with a known concentration of MBL as the opsonin source. Our data revealed that at high concentrations of MBL, uptake of GBS was increased. However, a decline in engulfment at lower concentrations of MBL suggested that there is a threshold level required for enhancement of GBS engulfment by human phagocytes. These results would appear to be in conflict with Van Emmerik et al (1994)

and Neth et al (2000) who report that MBL demonstrates little binding to GBS and that this low level of binding is not sufficient to make MBL an efficient opsonin for GBS. However, neither group examines phagocytosis to determine whether or not this level of binding to GBS can act as opsonization in a functional phagocytosis assay. Also, there is no indication as to the concentrations of MBL used in their binding assays and therefore it may have been below the threshold for enhanced uptake that we observed. It appears that higher levels of MBL may be needed to initiate opsonization with an organism like GBS that is highly encapsulated.

Our data from previous engulfment experiments, taken with the results of the MBL phagocytosis experiments, suggest that high concentrations of MBL in the sera of GBS-colonized women may lead to the increased engulfment of GBS by phagocytes from these women and that internalization by this pathway could facilitate the creation of a protective niche for GBS within the immune system.

One of the major events following phagocytosis of a microbe is production of cytokines that function to activate a variety of effector cells that initiate inflammation as well as the antigen-specific immune response. There are three primary pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6. These are secreted by the human immune system in response to GBS or various parts of the bacterium (De Bont et al, 1993; Williams et al, 1993; Peat et al, 1994; Vallejo et al, 1996; Von Hunolstein et al, 1997; Kwak et al, 2000; Berner et al, 2001). Because these cytokines can be found at high concentrations in the circulation of infants with GBS sepsis we were interested in investigating what effects these cytokines might have on GBS. Other studies have shown that cytokines enhance the growth and virulence characteristics of a variety of bacterial species (Denis et al,

1991; Denis and Gregg, 1991a; Denis and Gregg, 1991b; Porat et al, 1991; Hirsch et al, 1994; Meduri et al, 1999; Kanangat et al, 2001). Our data indicated that, of the three pro-inflammatory cytokines we tested, only IL-6 significantly enhanced GBS growth at a concentration that is physiologically relevant. We also determined that IL-6 biologic activity was required for its growth promoting characteristics. Similarly, Denis et al (1991) and Luo et al (1993) show that the biologic activity of IL-2 and TNF- α are necessary to exert their effects on *E. coli* and *S. flexneri*, respectively. We speculate that the requirement for the cytokine's biologic activity in enhancing GBS growth indicates that IL-6 may function as a signal for the bacteria that induces alterations in GBS virulence characteristics.

By using IL-6 to enhance its growth, GBS is again using the host's own machinery for its own benefit. IL-6 not only increases the bacterial load that the host must respond to, but may also decrease the availability of IL-6 for the host's own use. Other bacterial species, including *S. flexneri* (Luo et al, 1993), *S. aureus* (Kanangat et al, 2001), *Y. pestis* (Zav'yalov et al, 1995), bind host cytokines thereby potentially impairing the host's signaling pathways for activation of key effector mechanisms. We demonstrated that GBS binds to IL-6 and that this binding increases as the concentration of IL-6 is increased (Appendix D, Figure 1). We also demonstrated that the binding of GBS to IL-6 is a low affinity interaction based on the slow decline in IL-6 binding observed when unlabeled IL-6 was added as a competitor for the IL-6 binding site on the bacterial surface (Appendix D, Figure 2). The exact molecule on the GBS cell surface that interacts with IL-6 has yet to be identified. To date only the Caf1A usher protein of

Y. pestis has been identified as a surface protein that binds a host cytokine, IL-1 β (Zav'yalov et al, 1995).

As a way of approaching whether or not the IL-6 GBS interaction affects GBS virulence other than by increasing bacterial proliferation, we examined the gene expression of three of the most studied GBS virulence factors (the cytolysin, the capsule polysaccharide, and the C5a peptidase). Of the three virulence factors examined only one, the C5a-ase, showed an increase in mRNA levels following an eight-hour exposure to IL-6 and this increase was only detectable by RT-PCR analysis. It is possible that expression of these factors may be upregulated at other times after exposure. However, this is unlikely because the virulence factors produced by other bacteria are induced in the late exponential or stationary phases of growth (Chan et al, 1998; Winzer et al, 2001) similar to the point in the GBS growth cycle after 8 hours.

Because additional factors may be induced in response to IL-6 treatment of GBS, we took a global approach at examining induction of gene expression using random arbitrarily primed PCR. Of the four products we identified as being upregulated in response to IL-6, the most intriguing was the gene coding an ATP-binding protein that is associated with an ABC transporter. ABC transporters in bacteria function in many capacities including import and export of molecules involved in cellular metabolism, antibiotic resistance, and secretion of virulence factors (Higgins, 2001). Analysis of the genomic sequence surrounding this ATPase gene revealed that it was upstream from the GroES and GroEL genes. Real time PCR data indicated that not only was ATPase gene expression upregulated in response to IL-6, so was expression of the GroES and GroEL

genes. Western blot analysis also revealed that the increase in GroEL mRNA levels leads to an increase in GroEL protein.

The GroE proteins are heat shock proteins that are responsible for ensuring the proper folding of proteins that are destined to be secreted from the cell (Brocchieri et al, 2000). The GroEL protein has also been implicated in the pathogenesis of other bacterial diseases. The GroEL protein of *C. trachomatis* appears to be the antigen responsible for the delayed-type hypersensitivity response associated with chlamydial infections (Sanchez-Campillo et al, 1999). GroEL protein levels are increased upon contact with host cells or entry into macrophages by *L. pneumophila* (Garduno et al, 2001), *L. monocytogenes* (Gahan et al, 2001), and *M. tuberculosis* (Monahan et al, 2001). GroEL has also been identified as a mediator of attachment to host cells by *L. pneumophila* (Garduno et al, 2001), *H. influenzae* (Hartmann et al, 1997), *S. typhimurium* (Ensgraber et al, 1992), *H. pylori* (Phadnis et al, 1996) and *C. difficile* (Hennequin et al, 2001).

The increase in GroEL gene expression in GBS exposed to IL-6 suggests that IL-6 is capable of inducing a stress response in GBS that in some manner is similar to that which it would encounter in the host environment. Further investigation of the role of GroEL in the pathogenesis of GBS is warranted.

While there is still a great deal to learn concerning the virulence of group B streptococcus, the data presented here sheds some light on basic interactions between the host innate immune system and GBS. We have demonstrated that some components of the host immune defenses that should function to protect the host against GBS colonization or infection may actually contribute to the process. A clearer picture of

these host-bacteria interactions will assist in identifying additional targets for therapeutic and preventative strategies for GBS.

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APPENDIX D. ADDITIONAL FIGURES FOR CHAPTER 5

FIG. 1. An increase in GBS binding is observed as the concentration of FITC-IL-6 is increased. GBS was incubated with increasing concentration of FITC-IL-6 ranging from 0 – 20 ng prior to detection of binding by flow cytometry. From this curve, 10 ng FITC-IL-6 was used in the experimental binding assays.

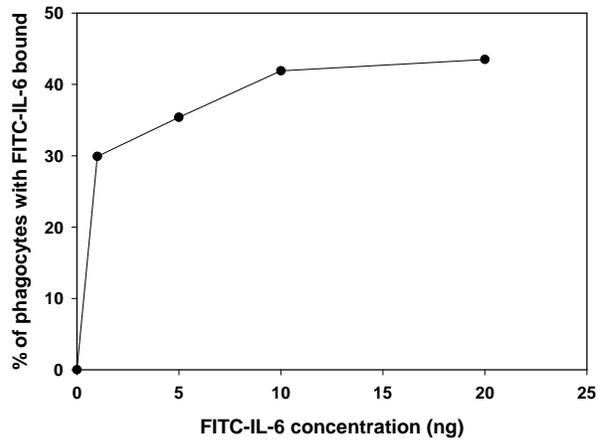


FIG. 2. Competition for the GBS IL-6 binding site between FITC-IL-6 and unlabeled IL-6 reveals that the interaction has low affinity. GBS was incubated with 10 ng FITC-IL-6 and concentrations of unlabeled IL-6 from 0 – 100 ng for 8 hours prior to detection of binding by flow cytometry. Error bars represent the range of values from two separate experiments.

