Receptors and ligands involved in the recognition of group B streptococci by human and bovine phagocytic cells

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University of New Hampshire, Durham

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Abstract
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The $\beta_2$ integrins, a heterodimeric family of leukocyte receptors, have been implicated in adherence of GBS to macrophages in the absence of opsonins. GBS bound to CD18-deficient bovine polymorphonuclear leukocytes (PMNs) almost as well as bovine PMNs with normal CD18 surface expression, suggesting that CD18 is not essential for adherence and that additional receptors are involved in adhesion. In soluble binding assays, three macrophage proteins (60, 55 and 20 kDa) were found to bind to GBS. The results were confirmed when the experiments were performed using Listeria monocytogenes. In competition assays, the organisms competed for attachment to U937 cells. In addition, Listeria bound to the same U937 proteins as did GBS. These results support the roles of proteins in the interaction of GBS and Listeria to macrophages.

Because adherence involves participation from the host and the bacteria the present study was expanded to investigate the role of GBS envelope proteins in the adherence to macrophages. The GBS adhesin for macrophages was identified using a soluble binding assay. A 21 kDa protein from GBS was shown to bind to U937 cells. Antisera to the 21 kDa protein was able to inhibit adhesion of GBS to U937 cells as measured using flow cytometry. This antisera was able to cross-react with a number of GBS strains and additional Gram positive bacteria, suggesting, this may be a conserved adhesin.

Keywords
Biology, Microbiology, Health Sciences, Immunology
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RECEPTORS AND LIGANDS INVOLVED IN THE RECOGNITION OF GROUP B STREPTOCOCCI BY HUMAN AND BOVINE PHAGOCYTIC CELLS

BY

L. MARY SMITH
Baccalaureate of Science, University of New Hampshire, 1989

DISSERTATION

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in

Microbiology

September, 1997
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29 August 1997
Date
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ABSTRACT

RECEPTORS AND LIGANDS INVOLVED IN THE RECOGNITION OF GROUP B STREPTOCOCCI BY HUMAN AND BOVINE PHAGOCYTIC CELLS

by

L. Mary Smith
University of New Hampshire, September, 1997

Macrophages have been shown to bind pathogenic microorganisms in the absence of exogenous opsonins, but the precise mechanisms are poorly understood. Microbial recognition by macrophages under these conditions becomes particularly important in individuals who have decreased amounts of opsonins such as immunocompromised individuals and neonates. This study was undertaken to investigate the mechanisms of adherence between group B Streptococcus and macrophages in opsonin-limiting conditions. Using several adherence assays; visual binding, enzyme-linked immunosorbent, and flow cytometry, GBS was able to adhere to U937 cells, a human macrophage-like cell line, in a dose-dependent manner without exogenous opsonins.

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support the roles of proteins in the interaction of GBS and *Listeria* to macrophages.

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INTRODUCTION

1. The macrophage

Cells of the monocyte-macrophage series constitute the mononuclear phagocyte system, which consists of circulating monocytes and tissue macrophages. These cells are found throughout mammalian tissues and function critically in the ecology of the body. The mononuclear phagocytic cells are phylogenetically one of the most primitive cell types. Cells with similar morphology and function are found in early life forms, and single-cell protozoa like the amoeba have considerable similarity to the macrophage (50). The mononuclear phagocytic cells arise from bone marrow myeloid stem cells, which no longer have the capacity to differentiate along the lymphoid pathway, and give rise to precursor cells for monocytes, erythrocytes, megakaryocytes and granulocytes (1). The myeloid stem cell gives rise to “committed” precursor cells that have extensive capacity for replication; however, these committed cells are restricted to maturation along one or at most two cell lineages. During their maturation these cells acquire the tools that equip them to phagocytose both foreign invaders such as pathogenic bacteria and endogenous invaders such as neoplastic cells (57).

Because macrophages are the main host defense until the development of the adaptive immune response it has been speculated that these cells may be multi-functional (153). In vertebrates a highly adaptive immune system has evolved involving extensive cooperation between cellular and humoral components and the concept of specialized recognition, and this facet of the host’s defense mechanisms has been the focus of comprehensive research (138). The macrophage has also been extensively studied; however, the precise mechanisms of attachment, intracellular signalling and engulfment of bacteria are poorly understood.
A phagocytic cell comes in contact with a variety of particulate materials, yet it attaches and ingests only selected particles. The cell must therefore be capable of selected recognition. The plasma membrane bears structures that recognize specific molecules on the surfaces of certain particles. Serum factors, termed opsonins, coat the surface of some bacteria and expedite the attachment process by macrophages (160). Specific antibody directed against a bacterial surface epitope is an opsonin that can bind to its epitope on the bacterial surface. The macrophage via its Fc-receptors can bind to the Fc portion of the antibody and draw the bacteria into close proximity (85). There are three distinct classes of Fc receptors designated FcγRI, FcγRII, and FcγRIII that bind to the Fc portion of the immunoglobulin (Ig) G molecule. These receptors provide a critical link between the phagocytic effector cells and the lymphocytes that secrete Ig (105). The Fc receptors are members of a large multigene family that mediates a series of diverse functions critical to cellular immune responses, including phagocytosis, antibody-dependent cellular cytotoxicity, secretion of inflammatory mediators, generation of the respiratory burst, and cellular clearance of immune complexes (105).

Complement is a second opsonin that is able to coat the surface of the bacteria and aid in efficient recognition by the macrophage. Complement activation may occur by either the classical or alternative pathways (53). Complement activation by the classical pathway is initiated by the formation of soluble antibody-antigen complexes or by the binding of antibody to antigen on a target, such as bacterial cells (57). The alternative pathway can be activated by the interaction of specific complement components and lipopolysaccharide or polysaccharides from certain bacteria. Regardless of which pathway initiates the process, activation of the C3 component is a key step in anti-bacterial host defenses. Cleavage of the C3 component yields C3a and C3b, C3b being the major bacterial coating component. Many bacteria undergo complement-mediated attachment to macrophages. Opsonization with C3 results in a striking enhancement of binding and phagocytosis of several microbial...
species such as *Leishmania major* (126), *Mycobacterium leprae* (134), *Rhodococcus equi* (64), *Legionella pneumophila* (119), and *Listeria monocytogenes* (30).

Macrophages have three distinct receptors, termed complement receptor (CR) 1, CR3 and CR4, that can recognize some fragment of C3. CR1, designated CD35, is the receptor for C3b and C4b (83). It is a polymorphic, N-glycosylated, monomeric protein with a molecular mass ranging from 190-250 kDa, depending on the allotypic variant (19). The role of CR1 in microbial clearance has received modest attention relative to its other functions. This receptor is able to bind ligands but does not stimulate internalization. However, treatment of cells with stimulators such as phorbol esters can convert CR1 into an endocytic receptor (19). CR1 can be converted from a binding site on resting cells to a receptor capable of transmitting the cellular signals required for internalization. It has been suggested that the main role of CR1 in microbial recognition may be as a cooperative receptor that facilitates both FcR- and CR3-mediated endocytosis (105).

The leukocyte integrin, CR3 (Mac-1, CD11b/CD18) binds to a variety of surface ligand including C3b and iC3b. CR3 is a heterodimer structure composed of a 95-kDa β chain (CD18) noncovalently associated with a 165-kDa α chain (CD11b). CD18 is common to two other related receptors, LFA-1 (CD18/CD11a) and p150/95 (CR4; CD18/CD11c). These three receptors are commonly called the leukocyte integrins. Phosphorylation of CD18 is thought to mediate the endocytosis of complement-fixed particles (105). CR4 has been implicated in the binding of the C3 fragments iC3b and C3dg. The biological role of CR4 remains unclear and awaits the identification of other ligands for this receptor, especially cellular ligands that may be involved in leukocyte adherence or migration (105).

There is ample and compelling *in vivo* evidence that complement is an important mediator of bacterial clearance by phagocytic cells and therefore is an important component of host defense. This is primarily accomplished through the action of CR3. The
importance of the leukocyte integrins to host defense is best illustrated by a congenital leukocyte adhesion deficiency disease in which patients express no or small amounts of β₂⁻ integrins because of a defect in the common CD18 gene. These patients suffer from a variety of frequently recurring, sometimes fatal, bacterial infections (6).

There is a large body of evidence that supports the concept of non-opsonin-mediated attachment and phagocytosis of certain bacteria. Early investigators hypothesized that either the net surface charge or hydrophobicity of a particle determined whether or not it could be recognized by phagocytic cells (38,107,123). Experimental evidence to support these hypothesis was difficult to obtain. Macrophages have the ability to bind to a wide variety of particles that differ considerably in their surface properties and modification of net surface charge or hydrophobicity does not necessarily affect ingestibility (84). Therefore, the macrophage must have specific receptors that recognize bacterial surface ligands independently of opsonization. Group B Streptococcus (GBS) (7,141), Mycobacterium tuberculosis (145), certain Salmonella species (3), and Escherichia coli (40) are all able to attach to macrophages in the absence of exogenous complement in in vitro assays. Some of the mechanisms for complement-independent attachment have been elucidated for certain bacteria.

For example, complement receptors can directly serve as the receptors for some bacteria in the absence of opsonins (7,40,43,124,141). In the case of GBS, experimental evidence has shown that blocking these receptors with monoclonal antibodies significantly decreases attachment, but does not abolish binding (7,141), suggesting that there are additional receptors involved in attachment. Even though complement receptors appear to play a major role in attachment of certain organisms, evidence is growing that other receptors on the surface of phagocytic cells play a significant role. Gbarah et al. (43) found that fimbriated E. coli bound exclusively to CD11/CD18 glycoproteins on SDS/PAGE-separated lysates of granulocytes. However, when the blots were overlaid with isolated
cross-linked fimbriae, the main component recognized by the probe was a 55-kD
glycoprotein, which is in the reported range of human Fc receptors. Further work with *E.
coli* has shown that the nonspecific cross-reacting antigen (NCA), a CD66 cluster antigen,
is a receptor for binding of bacteria expressing type 1 fimbriae (pili) (133). Results from
these studies indicate that *E. coli* expressing type 1 fimbriae binds to high mannose
oligosaccharide structures on NCA or/and a 55-kD protein.

Relman *et al.* (163) showed that *B. pertussis* binds to alveolar macrophages by two
receptors. Filamentous hemagglutinin from *B. pertussis* interacts with galactose-containing
glycoconjugates and the integrin CR3 in the absence of complement. *Mycobacterium
tuberculosis* has also been shown to use multiple receptors to adhere to macrophages.
Virulent strains of *M. tuberculosis* have been shown to use the phagocyte mannose receptor
and complement receptors CR1, CR3, and CR4 to achieve optimal binding and
phagocytosis (134).

Macrophages express scavenger receptors on their cell surface. These scavenger
receptors exhibit unusually broad binding specificity for polyanionic ligands and have been
implicated in various host defense functions. One of the principal functions of scavenger
receptors may be their participation in the clearance of microbes and highly toxic microbial
surface constituents from the body (121). A study by Dunne *et al.* showed that a secreted
form of the type 1 bovine macrophage scavenger receptor binds to intact Gram-positive
bacteria, including *Streptococcus pyogenes, Streptococcus agalactiae* (GBS),
*Staphylococcus aureus, Enterococcus hirae* and *Listeria monocytogenes* (31). This directly
implicates scavenger receptors in anti-microbial defense strategies.

Finally, a recent study by Elomaa *et al.* (34) described a novel protein expressed
on the surface of macrophages from the marginal zone of the spleen and the medullary cord
of lymph nodes that bound labeled bacteria and acetylated LDL, but not yeast or Ficoll.
The results suggest that the novel protein is a macrophage-specific membrane receptor with
a role in host defense, as it shows postnatal expression in macrophages, which are considered responsible for the binding of bacterial antigens and phagocytosis. There are, thus, a number of specific and non-specific interactions taking place between macrophages and bacteria that involve multiple structures from each. Elucidating the precise interaction between the macrophage and GBS is the goal of this research. To study this interaction I selected the cell line, U937.

2. U937 cell line

The U937 cell line was derived from a previously healthy 37-year old man with a generalized histiocytic lymphoma (146). The cell line was characterized by Sundström and Nilsson as similar to cells of the monocyte-macrophage lineage. The general characteristics of the cell line are found in Table 1. The specific distinctions of this cell line from that of lymphoid lineage are several-fold. For example, this cell line has the capacity to produce lysozyme which is normally secreted and synthesized by monocytes and macrophages. The cytochemical findings in U937 cells strongly favor a histiocytic origin (146). Phagocytic capacity is a feature of normal monocytes and U937 cells are phagocytic. The surface markers of C3 and Fc receptors are present on U937 cells as they are in normal monocytes and macrophages (146). This cell line is not able to produce Ig.

Minta and Pambrun (103) found that treatment of the immature U937 cell line with phorbol myristate acetate converts these cells to mature macrophage-like cells. After treatment the cells are no longer able to divide. They express properties characteristic of macrophages, such as the ability to undergo chemotaxis, the ability to produce superoxide anion, the presence of specific membrane receptors and the ability to phagocytose foreign agents. After extended periods of time these cells will revert back to the immature state and start dividing with the loss of the mature cell characteristics (65). Studies with U937 cells
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>U937 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
</tr>
<tr>
<td>Cell type</td>
<td>Monocytoid</td>
</tr>
<tr>
<td>Cell diameter (µm)</td>
<td>12.5, range 8.1-16.9</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Moderate variations in shape (round-ovoid)</td>
</tr>
<tr>
<td>Cell surface</td>
<td>Several bubble-like villi on most cells</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Irregular, lobated, large nucleolus in majority of cells</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Sparse ER. Small swollen mitochondria</td>
</tr>
<tr>
<td></td>
<td>Golgi apparatus well developed. Abundant vesicles. Moderate number of polyribosomes.</td>
</tr>
<tr>
<td>Nucleo-cytoplasmic ratio</td>
<td>high</td>
</tr>
</tbody>
</table>

**In vitro**

- Dependence on complete medium: Yes
- Dependence on feeder layer or conditioned medium: Yes
- Growth in clumps: No
- Attachment to feeder cells: A few cells attach loosely in old cultures
- Growth in agarose: No
- Population doubling time (h): 95
- Maximum cell density (cell/ml): $0.9-1.1 \times 10^5$

**Cytochemistry**

- Esterase activity strong; naphthol AS-D acetate esterases inhibited by NaF. β-glucuronidase present in moderate amounts

**Surface receptors (% positive cells)**

- SRBC: 0
- C3: 29
- Fc: 31

**Surface Ig**

- No

**Secretion of Ig**

- No

**Lysozyme production (µg/10^6 cells per 24 h)**

- 0.7

**β₂-microglobulin production (ng/5 x 10^6 cells/65 h)**

- 200

**Phagocytosis**

- Yes

**EBV genome**

- No

**Karyotype**

- Aneuploid

**Clonality**

- Monoclonal

---

Table 1: Characteristics of the U937 cell line from the original manuscript of Sundström and Nilsson (146).
have concentrated on the differentiation of these cells by particular agents; however, this
cell line has also been used to study the growth of intracellular pathogens, such as
*Legionella pneumophila* (14).

Because these cells originated from a lymphoid tumor they are able to propagate *in vitro* indefinitely. Since these cell are of human origin, they provide a good model for studying a human pathogen, such as GBS. Finally, compared to the commonly used peritoneal macrophage, U937 cells provide a homogenous population free of contaminating cells.

3. **Group B Streptococci**

GBS are facultative, β-hemolytic, Gram-positive cocci that commonly grow in long
chains (Figure 1). These organisms possess a distinct polysaccharide capsule that is
responsible for the serotype specificity (88). These organisms were first linked with
human disease when Fry reported three cases of puerperal sepsis due to this organism (41).
Currently, this organism is the leading cause of neonatal sepsis and meningitis, as well as a
major cause of maternal peripartum infections (chorioamnionitis, postpartum endometritis,
urinary tract infections and peripartum bacteremia) (11,157).

In newborns, two distinct syndromes of invasive GBS infections occur. Early-
onset disease is characterized by presentation of illness within the first week of life,
although most of these infections are evident soon after birth (2,113,128,136) . An
estimated 7600 cases of neonatal invasive infections due to GBS occur annually (164).
Early-onset disease has an incidence of 1.3 to 3.7 per 1000 live births and affects male and
female infants equally (113,128). The mean age of onset is 20 h, with close to half and
two-thirds of infants, respectively, becoming symptomatic within the 1st and 12th hours of
life, indicating that infection starts *in utero* in most of these infants (113). The majority of
affected infants are full-term; however, attack rates are highest among preterm
Figure 1. *Streptococcus* spp. Scanning electron micrograph of *Streptococcus* spp. Photo by Bryan Larsen.
infants. Birth weight is inversely related to infection risk. Bacteremia is almost always detected in early-onset disease. The three most common clinical presentations are sepsis without a focus, pneumonia, and meningitis (113,136). Mortality due to GBS disease has decreased from 50% in some reports from the 1970s to less than 10-15% in large population-based studies in the 1990s (2,113,136).

In early-onset GBS disease, the organism is acquired vertically by the infant from a mother who has vaginal or anorectal colonization either through ascending infection in utero just before delivery, through ruptured or intact membranes, or during passage through the birth canal (113,136). Approximately 50% to 75% of infants born to colonized mothers will acquire GBS vertically with only 1%-2% developing invasive GBS disease (113,136).

Late-onset disease usually occurs within the first 24 d of life. Late-onset has an incidence rate of 0.5 to 1.8 per 1000 live births (113). The most common clinical manifestation is typically meningitis (85% of the cases), but bacteremia without a focus has been reported to occur more often in recent years (113). The mortality rate has decreased from 25% in the 1970's to 10%; however, up to 50% of the survivors of meningeal infection have permanent neurologic sequelae (113). At least half of late-onset infections are acquired at birth from genitally colonized mothers and the rest are presumably acquired postnatally from the mother, the community or nosocomially (113).

Even though GBS infections are most common in the neonatal populations, adult infections due to this organism also occur. GBS infections in adults usually manifest as urinary tract infections, peripartum sepsis, soft tissue infection, osteomyelitis, infective endocarditis, or pneumonia (112). These infections are most common among individuals with certain underlying conditions such as diabetes, cancer, AIDS, cirrhosis and also individuals who are intravenous drug users, alcoholics or who are pregnant (36,72,132). GBS is a common isolate from the genital and the lower gastrointestinal tracts, although in
immunocompromised individuals it is considered an opportunistic pathogen (33). The annual incidence of invasive GBS infections in adults is approximately 4.4 per 100,000 (36). In a population-based assessment study Farley et al. concluded that 67% of all deaths related to GBS infections that occurred during the study occurred in men and nonpregnant women (36). In this study they found that invasive GBS infections were twice as high in black adults as in whites, and was particularly high in older black adults. These associations may be due to socioeconomic factors (36). In another study GBS were found to infect both sexes equally (132). However, among older populations, males were much more commonly affected. The reasons for male predominance in this age group is unclear but may be related to the frequency of obstructive uropathy in older males (132).

Isolates of GBS can be classified into seven well-defined capsular serotypes (Ia, Ib, II, III, IV, V, VI) and at least two additional provisional serotypes (157). Available data concerning capsular type and other phenotypic markers of strains associated with either vaginal or anorectal carriage in women indicate that types Ia, Ib, II and III are the most common; additionally, these strains account for 90%-99% of invasive neonatal disease (157), with type III being the most common (125). However, it appears that a different pattern is emerging in adults. A recent study conducted by Harrison et al. (58) investigated recurrent infection in adults with invasive GBS infection and showed that over 40% of the population studied was infected with serotype V. In adults type V is the most common isolate followed by Ia, Ib, II and III. There have been cases of infants infected with serotype V invasive GBS in early-onset disease, indicating that this new apparently adult virulent strain can cause disease infants (125). A study in Spain indicated that type V was the third most common strain of invasive GBS in neonates (62), which may indicate that this recently identified strain is becoming more prevalent. Previous studies defining the distribution of serotypes among GBS isolates from neonates with invasive disease were established more than a decade ago (125). This would indicate that additional studies need
to be performed to investigate the prevalence of type V and the redistribution of the other strains in GBS disease in both adults and infants.

4. Virulence Mechanisms

Because GBS is believed to be transmitted from the colonized mother to the infant upon passage through the birth canal, studies have been conducted to investigate the factors influencing adherence of GBS to human vaginal epithelial cells. One study indicated that there was a significant difference in adherence capabilities among four GBS serotypes, with serotype III strains exhibiting the greatest adherence ability (20). However, another study conducted at the same time showed no difference in the adherence potentials of the different serotypes and neither group-specific antigens, nor type-specific antigens played a role in adherence (165).

A. Capsule

The type III strain of GBS has accounted for the majority of neonatal GBS disease and has been the focus of major research. The type III capsular polysaccharide is a repeating pentasaccharide subunit consisting of a glucose, galactose, and N-acetylglucosamine backbone with a disaccharide side chain composed of a terminal sialic acid residue attached to a galactose. The structure of the capsular polysaccharide is important for its function as a virulence factor (66). Early studies showed that the GBS capsule was responsible for virulence. In studies by Durham et al. (32) the quantity of polysaccharide released into the growth medium in vitro correlated with bacterial virulence in vivo. A study by Levy et al. (91) indicated that soluble GBS polysaccharide inhibits complement-mediated opsonophagocytic killing of GBS in human sera. Rubens et al. (130) developed an isogenic mutant of GBS type III using transposon mutagenesis, which no longer expresses surface capsule. They found that the unencapsulated GBS was less
virulent in a neonatal rat model then the wildtype parent strain indicating that capsule expression is important for GBS virulence in vivo. A later study by Wessels et al. (156), using transposon mutagenesis to create a GBS strain that was deficient in the terminal sialic residue in the capsule, showed that this sialic residue was important in the virulence of GBS. This strain was less virulent than the wild-type parent in a rat neonatal model. The presence of sialic acid residues on the surface of a bacterial cell maybe an important virulence mechanism for GBS to avoid killing by macrophages, but it may not be as important in invasion. A study by Hulse et al. (66) showed that the capsule does not have a role in a receptor-ligand interaction involved in the mechanism of GBS entry into respiratory epithelial cells. It was found that unencapsulated organisms adhere to and invade epithelial cells better than their encapsulated counterparts. GBS were able to regulate capsular expression according to the growth phase of the organism as well as to the nutrient availability in the environment (13). In vivo modulation of type III GBS capsular sialic acid or polysaccharide may contribute to virulence by promoting intracellular invasion with reduced expression or evasion of host defenses with increased expression (47).

B. Lipotechoic acid

Teichoic acids are cell wall polymers that are found in most Gram-positive bacteria. Teichoic acids have been described as polymers of glycerol phosphate or ribitol phosphate which are substituted by ester-linked alanine and often with glycosyl residues (111). However, as the result of examination of several Gram-positive bacteria, it became apparent that many variations in these basic structures existed; therefore, the definition has been expanded to include all surface-associated polymers containing glycerol phosphate or ribitol phosphate (155). Thus, the term teichoic acids refers to those polymers that are covalently linked to peptidoglycan (wall teichoic acid), as well as those that are membrane-
associated (lipotechoic acid; LTA) (111).

LTAs are believed to play an important role in several diseases by mediating the attachment of pathogenic organisms to host cells. Nealson and Mattingly (111) found that GBS contains LTA and deacylated glycerol teichoic acids that are cell-associated. These LTA structures are polymers (glycerol phosphate) that are linked to lipid moieties that presumably serve to anchor the molecules in the cytoplasmic membrane. GBS also produce cell-associated glycerol teichoic acids, and they demonstrated that this material is likely deacylated LTA and not wall teichoic acids, which is covalently linked to peptidoglycan (111). They also found that all serotypes of GBS isolated from infants with early- or late-onset disease possessed very high levels of LTA. In contrast, GBS strains isolated from infants without disease and the avirulent control strain contained low levels of cell-associated LTA (111). These studies suggest the importance of LTA as a virulence mechanism in GBS disease.

In a later study Nealson and Mattingly (109) found that LTA of GBS serotype III appeared to be the major cell-associated material responsible for attachment to human embryonic, fetal, and adult epithelial cells; however, adherence of virulent strains containing high levels of LTA bound better to embryonic and fetal cells than adult cells. After the initial hydrophobic interactions by GBS, secondary binding possibly involves the interaction of the glycerolphosphate backbone of LTA with the embryonic and fetal cell surfaces (110). These cell types appear to possess receptor sites primarily composed of glycoproteins. These conclusions were based on experimental evidence showing that binding of GBS type III to embryonic and fetal cells pretreated with sodium periodate or trypsin was significantly decreased. During early development receptor components on these cell surfaces may differ from those on adults cells, because similar pretreatment of adult cells with trypsin or sodium periodate did not alter the binding efficiency (110).

It thus appears that the expression of LTA on GBS affects the ability of this
organism to bind to host cells. Recent investigations have shown that GBS secrete LTA after exposure to penicillin (98). Thus, large numbers of organisms metabolizing on the meninges or mucosal surfaces and secreting LTA could aid in host tissue destruction by secreting this polymer directly onto host cell surfaces (51).

C. Proteins

Proteins account for approximately 40% of the GBS cell wall weight (27) and treatment of certain strains of GBS with pepsin or trypsin greatly abrogates the binding of these strains to host cells (98,151). Cell wall-associated proteins from GBS may thus be an important virulence mechanism that aid in the adhesion to host cells. A study by Goldschmidt and Panos (51) showed that a strain of GBS with only a very small amount of surface of LTA adhered to a human cell line (HeLa), but not by an LTA-mediated mechanism, suggesting a protein-mediated attachment. A study by Miyazaki et al. supports this conclusion. They showed that a protein factor rather that LTA was primarily responsible for attachment to young, synchronously growing, subconfluent eukaryotic monolayers in tissue culture (104). As in previous studies, they used a proteolytic enzyme, in this case, pronase, that drastically decreased binding. Heat completely abolished binding, suggesting protein involvement in adhesion.

Some of the surface proteins on GBS have been characterized. Lancefield et al. showed that antibodies raised to proteins currently known as C-proteins provided passive protection to mice challenged with GBS; this passive protection did not extend to non-C-bearing stains (89). C-proteins have been found on all serotype Ib and many type Ia, and II strains of GBS, but they are uncommon on type III GBS. The molecular mass of C-proteins ranges from 14 kD to several hundred thousand daltons (101). Two different C-proteins have been described. The α antigen is resistant to the protease trypsin and does not bind Ig (17,159). The β antigen is sensitive to both trypsin and pepsin and binds to the
Fc portion of human IgA (22,131). The association of C-protein antigen with virulence in human has not been clearly defined, but it has been reported that serotypes with this antigen are found in association with adult meningitis (158). In addition, strains of serotypes Ib and Ic, both possessing the C-protein antigen but different type polysaccharide antigens, constitute approximately one-third of the types prevalent in colonized mothers, colonized asymptomatic newborn infants, and newborn infants with early-onset of bacteremia or sepsis (73).

Strains that possess the C-protein antigen have been shown to have enhanced resistance to opsonophagocytosis and intracellular killing by phagocytes (120) *in vitro*, adding to their potential role in virulence. Madoff *et al.* (96) showed that α-positive strains are more resistant to opsonization in the absence of antibody than α-negative strains. In the presence of α-specific monoclonal antibody, the opsonophagocytic killing of GBS strains is dependent on the quantity of antigen expressed and on the molecular weight of the largest immunoreactive band of α-C-protein (96). In their study 90% of the clinical isolates tested contained α-C-protein (either alone or in combination with β-C-protein), whereas 42% contained the β-C-protein (either alone or with α-C-protein). This would suggest that immunotherapeutic treatment should be targeted at the α-C-protein (96).

Since the role for C-protein has been shown to be important in immunity against GBS, Michel *et al.* determined if antisera against the proteins could be protective in mouse models. They purified the β-C-protein and used it to generate antisera in rabbits. This antisera was given to pregnant mice. The neonatal pups were then challenged with a strain of GBS expressing β-C-protein; 68% of these pups were protected by the immune antiserum, whereas no controls were protected (97). The immune serum facilitated opsonophagocytic killing of GBS strains possessing the β-C-protein, but not those strains that do not express the antigen. In the same study female mice were immunized with the rabbit antiserum 2 months prior to mating. One- to two-day offspring of these dams were
challenged with GBS and were protected in a dose-dependent manner, with 96% survival in the high-dose group and 20% survival in a sham control group. Thus, active immunization of mice with the GBS β-C-protein confers protection against lethal infection with β-containing GBS to their offspring (97).

Another surface protein from GBS has been shown to be important in protection studies. R protein is widespread among human GBS isolates. Lindèn et al. (94) found that 51% of type II and 87% of type III GBS carry R protein whereas the antigen is not present among type Ia and Ib. In mouse protection studies, they found that antibodies against R-protein protect mice from challenge with type II GBS carrying this protein, but not type III GBS (93). The reason for this discrepancy is unclear. One possibility is that the sialic acid of the type III polysaccharide prevents anti-R antibody from facilitating bacterial opsonization (93).

GBS also contain a surface protein termed R4 protein. Western blot analysis was used by Fasola et al. (37) to determine the prevalence of antibodies against the R4 antigen in mothers colonized with GBS serotype II and III with and without expression of surface-localized R4 antigen, as well as the concordance of the presence of antibodies to R4 in GBS-colonized mothers and their infants. They found that the R4 protein antigen induces an immune response in the host and that there is transplacental passage of these antibodies. This suggests that the R4 protein may be a potentially important component of a GBS polysaccharide-protein conjugate vaccine to induce type-specific immunity in mothers (37).

D. Intracellular Survival

A recent study conducted by Valentin-Weigand et al. (151) investigated the interactions of GBS with macrophages to compare the uptake and survival of nonopsonized and opsonized GBS. They found that GBS can enter macrophages in the absence of opsonins via a mechanism involving both microfilament-dependent phagocytosis and
receptor-mediated endocytosis. In addition, they found that opsonin-independent entry of GBS results in intracellular survival that is significantly enhanced compared with that resulting from opsonin-dependent entry. This may be due to the fact that entry in the absence of opsonins leads to a decreased activation of the antimicrobial activity of these macrophages (151). GBS were able to survive intracellularly for more than 24 hours, which in vivo would be sufficient time to maintain the bacteremia required for meningitis. GBS were found inside the phagosome, suggesting that nonopsonized GBS must have been able to evade intraphagosomal antibacterial activities. It is possible that GBS were able to inhibit phagolysosomal fusion.

Although there is much known about the interactions between bacteria and macrophages the precise mechanisms involved in initial adherence are poorly understood. There appears to be cooperative involvement between the bacteria and the macrophage. Their relationship is a dynamic one. This notion is supported by work from Hasty et al. (60). Figure 2 shows a hypothetical two-step model for the interaction of streptococci with host cells. This model suggests that there are at least two sequential steps, probably involving at least two different adhesins, that lead to firm adhesion. The proposed first step is a weak interaction between LTA and a receptor on the host cell. The second interaction in this model is a much stronger one that involves a bacterial protein interacting with its receptor on the host cell (60). Even though this model is derived from results obtained with S. sanguis and S. pyogenes, its principal features should be valid for any streptococcal species (60).

Understanding the mechanism of attachment for GBS and macrophages is the goal of this dissertation. Initial work in our laboratory focused on the macrophage and its involvement in adherence. It was found that GBS can attach to macrophages via the CR3 receptor (141) in the absence of opsonins; however, 100% inhibition was never achieved. This is supported by additional research (7). Therefore, these studies have focused on
Figure 2. **Multi-step adhesion model.** Hypothetical two-step model for the interaction of streptococci with host cells. This model was proposed by Hasty et al. (1992).
identifying other surface proteins from the macrophage involved in bacterial attachment. In the above discussion on GBS surface proteins it is apparent that certain proteins can have a role in the virulence mechanisms of the bacteria; however, to date no one has investigated the role of GBS proteins in attachment to macrophages. This study also investigated if proteins from GBS are directly involved in adhesion to macrophages.
CHAPTER I

GROUP B STREPTOCOCCI BIND SPECIFICALLY TO A U937 CELL SURFACE PROTEIN IN THE ABSENCE OF EXOGENOUS COMPLEMENT

ABSTRACT

The mechanisms of attachment of group B streptococci (GBS) to host cells was investigated in this study. The susceptible population to GBS infection may be deficient in opsonin production, so this study was undertaken to investigate the receptors on phagocytic cells that may be involved in binding GBS in opsonin-poor environments. GBS bound to U937 cells in a dose-dependent manner as measured by visual binding assays, enzyme-linked immunosorbent assays and flow cytometric assays. β2-integrin receptors on the surface of U937 cells were partially involved in the attachment process. GBS binding to U937 cells was inhibited by up to 57% by anti-CD18 antibody, 40% by anti-CD11b antibody, and 20% by anti-CD11c antibody. However, 100% inhibition was not achieved. This suggests a role for additional U937 cell receptors in the attachment of GBS. Three proteins with molecular masses of approximately 60, 55, and 20-kDa were found on the surface of U937 cells that directly bind to GBS in soluble binding assays and Western blot analyses. The 55-kDa protein has a pI of 5 as determined by two-dimensional gel electrophoresis. It is proposed that these proteins are also involved in attachment of GBS to U937 cells.

INTRODUCTION

For the past three decades, GBS have been the predominant bacterial pathogen in
neonates and young infants (55), accounting for the majority of cases of bacterial sepsis and meningitis (118). GBS have also been recognized as the cause of certain adult infections in individuals with compromised immune systems (118). The exact mechanisms by which GBS attach to host cells are not known. Therefore, the goal of this project was to investigate the role of surface proteins from phagocytic cells in the recognition of GBS.

Previous research had shown that GBS are killed by phagocytic cells only after opsonization with complement and, in most cases, type-specific antibody (12,120). More recently, however, investigators have shown that GBS can bind to phagocytic cells in the absence of complement and specific antibody (7,141). In fact, a portion of the population most susceptible to GBS infection may be deficient in complement and antibody. In these individuals nonopsonic phagocytosis may be a major host defense mechanism against GBS infection (148).

Phagocytic cells including macrophages and neutrophils have a number of receptors on their cell surface that have been implicated in binding bacteria. The $\beta_2$-integrins are a family of structurally and functionally related glycoproteins that exist on the cell surface as dimers (99). These molecules bind complement components on opsonized bacteria and increase phagocytosis by the host cell. However, recent evidence has shown that these receptors are also able to bind nonopsonized bacteria directly (7,141). CD14 is a 55-kDa protein found on the surface of phagocytic cells and has been shown to be a receptor for lipopolysaccharide, found on the surface of Gram-negative bacteria (162). This molecule has also been shown to bind to Gram-positive bacteria (86) and may potentially be involved in bacterial attachment. In the case of GBS, no phagocytic cell receptor has been identified that directly binds these bacteria.

GBS is normally acquired by the infant via the lungs; therefore the alveolar macrophages are likely to play a role in early host defenses. Neonatal alveolar cells are difficult to obtain and there is no human infant alveolar cell line commercially available;
therefore U937 cells were used. The U937 cell line was derived from a human histiocytic lymphoma and was characterized by Sundström and Nilsson (146). This cell line has many characteristics of normal macrophages (see Introduction). This cell line was chosen because it is easy to propagate and multiplies rapidly. In addition, this cell line not only expresses the β₂-integrins, but also CD14 (68) on its cell surface, which allow for easy study of these receptors. Treatment with phorbol esters causes the cell line to differentiate into mature macrophages (103) and to increase surface expression of the aforementioned receptors. This cell line has been employed in similar studies to investigate the growth of the intracellular pathogen Legionella pneumophila (14).

In this study the involvement of β₂-integrins and CD14 on the attachment of GBS to U937 cells was investigated. In addition, this study was performed to determine if there are additional surface proteins on U937 cells that play a role in adherence of GBS in the absence of opsonins.

MATERIALS AND METHODS

Bacterial Strains. GBS type III, designated COH31, was provided by Dr. Dennis Kasper, Channing Laboratory (Harvard University). This strain is a clinical isolate from an infant with meningitis (75). The bacteria were maintained as a frozen stock in Todd Hewitt broth (THB) (Difco Laboratories, Detroit, MI) with 10% dimethyl sulfoxide (DMSO). For bacterial studies, the frozen stock was thawed and streaked on a blood agar plate. A single colony was inoculated into 10-ml of THB and the culture was incubated overnight at 37°C. One milliliter of this overnight culture was used to inoculate a 10-ml tube of THB and this was incubated for 2-h at 37°C. This yielded approximately 10⁹/ml GBS as determined by colony forming units. The bacteria were grown fresh for each assay.
Cell lines. U937 cells (ATCC CRL-1593.2) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in Dulbecco's minimal essential medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS), penicillin/streptomycin (Gibco, Gaithersburg, MD) and L-glutamine (Gibco). This cell line was stored at -70°C as a frozen stock in FBS with 10% DMSO.

L929 cells (ATCC CCL-1), a mouse fibroblast cell line, served as a negative control for flow cytometry and ELISA assays. This cell line was maintained as a frozen stock in our laboratory. In culture, the cell line was grown as described above for the U937 cells.

Hybridoma cell lines were also maintained in our laboratory as frozen stocks. Hybridoma cell lines ATCC TIB-128 M1/70.15.11.5 (anti-CD11b), ATCC TIB-218 M18/2.a.12.7 (anti-CD18) and ATCC HB-247 60bca (anti-human CD14) will be designated as M1/70, M18/2 and 60bca respectively. They were grown as described for the U937 cells.

Antibody Production and Purification. The hybridoma cells were grown to terminal cultures in T-75 flasks (Costar, Fisher, Springfield, NJ) and the supernatant fluids were collected. The supernatant fluids were centrifuged at 10,000 x g for 30 min to pellet the hybridoma cells. The antibody-containing supernatant fluids (in the case of M1/70 and M18/2) were used directly for the ELISA inhibition assay. The 60bca antibody was partially purified using saturated ammonium sulfate. The saturated ammonium sulfate was added to the supernatant fluid at 45% and the antibody was precipitated overnight at 4°C. The precipitated antibody was collected with centrifugation at 20,000 x g for 1 h. The pellet was resuspended in 20 ml of phosphate-buffered-saline (PBS). The resuspended pellet was dialyzed at 4°C against PBS (1 L) with 3 buffer changes. The antibody
concentration was determined spectrophotometrically at OD$_{280\text{nm}}$.

**Adherence assays.** Adherence of GBS to U937 cells was measured using 3 assays.

a) **Visual binding assay.** U937 cells (1 x $10^7$) were incubated with phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) ($10^{-8}$M) in dimethylsulfoxide (DMSO) (Sigma) for 24 h in unsupplemented DMEM. Treated U937 cells were washed 3x with PBS to remove PMA. The cells were adjusted to 3 x $10^5$ cells/ml and 300 µl of the cell suspension was added to each well of 8-chamber glass slides™ (Lab-Tek®, Nunc, Inc., Naperville, IL). The plates were incubated for 2 h to allow the U937 cells to attach. A 10-ml, 2-h culture of GBS was washed 3 x in PBS and adjusted to $10^8$ cells/ml. The bacteria were incubated for 30 min at 37°C with the U937 cells at concentrations of $10^7$, $10^6$, and $10^5$ cells/well. Unbound bacteria were removed with 3 washes of PBS. The cells were stained with differential stain and bacterial attachment was quantitated microscopically. U937 cells with 2 or more bacteria attached were counted as positive. A total of 100 U937 cells were counted for each bacterial dilution. The samples were run in duplicate. A control with U937 cells containing no bacteria was also used.

b) **Enzyme-linked immunosorbent assay (ELISA).** Both PMA-treated U937 cells and untreated U937 cells (1 x $10^5$ cells/well) were plated into separate 96-well ELISA plates (Immunolon; Dynatech, Chantilly, VA). L929 cells were also plated into a 96-well ELISA plate and served as a negative control for GBS attachment. The cells were dried overnight at room temperature. The microtiter wells were blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. A 2-h culture of GBS was washed 3 x with PBS and reconstituted in 1M NaCO$_3$, pH 9.0. Biotin (Pierce, Rockford, IL; 0.1 mg/ml) was added to the bacteria and incubated for 1 h in the dark with gentle rocking. The bacteria were washed 4 times with PBS to remove unbound biotin. The labeled-
bacteria, at various concentrations, were added to the assay wells containing U937 cells and the plates were incubated for 2 h at room temperature. The unbound bacteria were removed with 3 washes of PBS. The bound bacteria were detected with streptavidin (SA) conjugated to horseradish peroxidase (HRP) (1:2500) (Pierce) in 1% BSA/PBS, with a 2-h incubation. The SA-HRP was removed with 3 washes of PBS. Fifty microliters of the substrate, 2,2' Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS)(Sigma), plus 1% hydrogen peroxide (H₂O₂) was added to each well and incubated for 15-30 min. Bacterial attachment was measured using a microplate reader (Bio-Rad, Hercules, CA) at OD _405nm_.

c) Flow Cytometry. Approximately 5 x 10⁵ U937 cells (both PMA-treated and untreated) in PBS were added to 5-ml Falcon tubes (Fisher). In addition, L929 cells were used as a negative control for the flow cytometry assay since GBS do not adhere with any significance to this cell line. A 10-ml, 2 h culture of GBS (COH31) was grown in THB and washed with PBS. The cells were labeled with fluorescein isothiocyanate (FITC); (1-mg/ml in 1.0 M NaCO₃, pH 9.0) and incubated for 1 h at room temperature, with gentle rocking in the dark. The cells were washed 4 times with PBS to remove unbound FITC. The bacteria were added to the U937 and L929 cells at various concentrations. A control sample included U937 and L929 cells containing no bacteria to determine autofluorescence. The cells were incubated for 1 h at room temperature and washed 3 x with PBS. The cells were fixed with 0.5% paraformaldehyde (Sigma). The samples were read on a Facscan™ flow cytometer (Becton Dickinson) and the results were analyzed using the Lysis II™ software. The results are represented as the percentage of U937 cells with attached GBS. Results are also represented as the mean fluorescence intensity of GBS attachment to U937 cells.

**Inhibition assays.** Two types of inhibition assays were performed. The first was an
ELISA assay. The ELISA wells were coated with U937 cells and the wells were blocked as described above. The monoclonal antibody supernatant fluids for M1/70 and M18/2 were added at various dilutions and incubated with the U937 cells for 2 h at room temp. A purified anti-CD11c designated BU15 (CR4 alpha chain, p150/95) (AMAC, Westbrook, ME) was also incubated with U937 cells at various concentrations. The unbound antibodies were removed with 3 washes of PBS. GBS was added at a dilution of $10^8$ cells/well. The plates were incubated for 2 h at room temperature. The bound GBS were detected with antiserum against whole GBS at a dilution of 1:5000. This was incubated for 2 h at room temperature. Unbound antibody was removed with 3 PBS washes and goat anti-rabbit conjugated to horseradish peroxidase (HRP) (Pierce) was added at a 1:7500 dilution and incubated for 2 h at room temperature. A control of U937 cells with GBS alone was used to measure maximal attachment. The assay was completed as described above. Inhibition was calculated using the following formula:

$$100 - \left( \frac{\text{OD}_{405\text{nm}} \text{ average of test wells}}{\text{OD}_{405\text{nm}} \text{ average of control wells}} \times 100 \right) = \% \text{ inhibition.}$$

Inhibition was also measured using flow cytometry. Anti-CD14 antibody (60bca) was added at various concentrations to tubes containing U937 cells and the mixtures were incubated for 30 min at room temp. The unbound antibody was removed with 3 PBS washes and FITC-labeled GBS were added to the U937 cells and incubated for 30 min at room temp. The assay was completed and the data were analyzed as previously described.

**Statistical Analysis.** All statistical analysis was performed using the computer software Statview™ version 4.5. Student T-tests were performed for flow cytometry anti-CD14 inhibition data.
Protein Identification. Enriched membrane fractions of U937 cells were prepared. U937 cells were treated with PMA and washed as previously described. The U937 cells were pelleted and 1.0% Triton X-100, 0.1% SDS in PBS was added to the pellet. The pellet was frozen at -70°C overnight. The suspension was thawed and the nuclear debris was removed with centrifugation (20,000 x g). Laemmli sample buffer (87) (100 µl) was added to the remaining supernatant fluid and the mixture was boiled for 1 min. The U937 cell proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (87) using a Hoefer™ gel system (Hoefer Scientific Instruments, San Francisco, CA). The separated proteins were visualized with Coomassie blue.

Identification of U937 cells surface proteins. Prior to membrane preparation the surface proteins of U937 cells were labeled with biotin (100 µg). Since biotin is unable to traverse the cell membrane (21) only the surface proteins are labeled. The U937 cell/biotin mixture was incubated for 1 h at room temperature with gentle rocking. The cells were washed 3 x with PBS to remove unbound biotin and membrane fractions were prepared as previously described (see above). The samples were separated on SDS-PAGE and the proteins transferred to nitrocellulose as described by Towbin (150) using a Biorad™ transfer unit. The nitrocellulose was blocked with 5% skim milk buffer (Difco) for 1 h. After blocking, the nitrocellulose was incubated with avidin-HRP (Biorad) (1:1000) in 1% skim milk buffer for 1 h with gentle rocking. The nitrocellulose was washed 3 times for 5 min each in PBS. The biotin-labeled surface proteins were visualized using 4-chloronapththol (Biorad).

Identification of U937 proteins that specifically bind GBS. Unlabeled U937 cell membrane preparations were separated on SDS-PAGE and transferred to nitrocellulose as previously described. After the nitrocellulose was blocked, 10^{10} GBS were added to the
nitrocellulose and incubated for 1 h at 37°C. Unbound bacteria were removed with 3 5-min PBS washes. Bound bacteria were detected using goat anti-GBS antiserum (1:1000). The antiserum was incubated with the nitrocellulose for 1 h at 37°C. Unbound antiserum was removed with three 5-min PBS washes and the U937 cell proteins with bound GBS were visualized with chemiluminescence (Amersham, Arlington Heights, IL).

**Soluble binding assay.** THB (10 ml) was inoculated with 1 ml of an overnight culture of GBS and the culture was this incubated at 37°C for 2 h. The washed bacterial pellet was reconstituted in 5% gelatin in PBS and incubated at 37°C for 2 h. Concurrently, 200-μl of biotin-labeled U937 membrane preparations were added to 800-μl of 5% gelatin, 1% Triton X-100, 0.1 % SDS in PBS and incubated at 37°C for 2 h. Experimental systems, containing GBS and U937 cell membrane preparations, and control systems, containing GBS and buffer, were incubated for 4 h at 37°C with end-over-end rocking. The bound U937 cell surface proteins were eluted from washed bacteria with 50 μl of Laemmlı sample buffer and boiled for 1 min. The mixture was centrifuged at 12,000 x g and the supernatant fluid containing the eluted proteins was retained. Ten microliters of the isolated protein suspension and of the control were separated on 12% SDS-PAGE (Mini-Gel, Biorad™). Proteins were transferred to nitrocellulose, blocked, and incubated with avidin-HRP as described above. The isolated U937 cell proteins that bound to GBS were visualized with chemiluminescence.

**Two-dimensional gel electrophoresis.** Two-dimensional gel electrophoresis (2-D gel) was performed as described by O’Farrell (114,115). Briefly, the first dimension tubes gels were prepared by mixing acrylamide, NP40, Milli Q H₂O and urea without ampholines. The ampholines (pH range of 3-10; Sigma, and 5-7; Biorad) were added fresh to the above mixture which was warmed to 30°C to dissolve urea. After degassing,
ammonium persulfate and TEMED were added to the solution and the solution was added directly to acid-washed glass tubes. The gels were allowed to polymerize overnight. The unlabeled U937 cell membrane samples were prepared as previously described. β-mercaptoethanol (4 μl) was added and the preparations were incubated on ice for 10 min. These membrane preparations were added to the tube gels. The bottom chamber of the 2-D gel apparatus was filled with 0.01 M H$_3$PO$_4$ and the top chamber, with 0.02 M NaOH. The samples were electrophoretically separated at 400 V for approximately 18 h.

Following electrophoresis, the gels were removed from the tubes and placed in Laemmli sample buffer. The second dimension gel consisted of a 12% SDS-PAGE slab gel (3mm) with a wide toothed comb. The tube gel was laid on the slab gel and anchored in place using 1% agar. The proteins were separated as previously described. Two gels were prepared simultaneously. One gel was stained with Coomassie blue to visualize the separated proteins; here the first dimension was run with ampholines in the pH range of 3-10. This range was used to observe all the U937 cell proteins. The other gel was used to transfer proteins to nitrocellulose; here the pH range of the ampholines used was 5-7. The tighter molecular weight range used in the second part of the assay was due to the fact that most membrane proteins are found in this pH range (5). The nitrocellulose was probed directly with GBS as previously described. The U937 cell proteins that were bound with GBS were visualized using chemiluminescence.

RESULTS

GBS attach to U937 cells. Three separate assays were performed to measure attachment of GBS to U937 cells in the absence of exogenous complement. The visual binding assay was quantitated microscopically. Figure 1a is a photograph of one U937 cell with 3 separate diplococci of GBS attached. One hundred cells were counted for each

30
Figure 1a. U937 cell with GBS attached. A visual binding assay was performed and the resulting photo represents one U937 cell with GBS attached. The cells were visualized using a differential stain.
bacterial dilution and the assay was performed in duplicate. Figure 1b shows the results from two separate experiments. Attachment of GBS to U937 cells occurs in a dose-dependent manner.

In the ELISA assay both PMA-treated and untreated U937 cells were dried to the microtiter wells. This prevents phagocytosis, which can interfere with measurements of adherence. The results are shown in Fig. 2a and indicate that, as with the visual binding assay, GBS bind to U937 cells in a dose-dependent manner. Treatment of the U937 cells with PMA did not significantly increase the binding of GBS to these cells, suggesting that the receptor on the U937 cells for GBS is not upregulated by this treatment.

Figure 2b shows the results from flow cytometry experiments. Again, the bacteria bound to U937 cells in a dose-dependent manner. Treatment with PMA did not increase attachment. Thus, all three procedures for measuring bacterial attachment to U937 cells yielded comparable results.

**β₂-integrins are involved in GBS attachment to U937 cells.** β₂-integrins have been implicated in the attachment of organisms to phagocytic cells even in the absence of opsonins. The inhibition experiments were designed to determine if blocking the β₂-integrin receptors affected the attachment of GBS to U937 cells. Figure 3a shows that, when U937 cells were treated with various dilutions of anti-CD18 antibody, GBS attachment was inhibited in a dose-dependent manner. When undiluted supernatant fluid of anti-CD18 was added to U937 cells there was greater than a 55% inhibition of GBS attachment. As the antibody dilution was decreased the inhibition was decreased. Figure 3b shows that when U937 cells were treated with various dilutions of anti-CD11b antibody that GBS attachment was also inhibited in a dose-dependent manner. The maximal inhibition of binding achieved with this antibody was 40%. Figure 3c shows the results when U937 cells were treated with various dilutions of anti-CD11c antibody. In this case,
Figure 1b. Visual Binding Assay. Two separate visual binding assays were performed in duplicate. The results are expressed as the percentage of U937 cells that have 2 or more GBS attached.
Figure 2a. ELISA measurement of attachment of labeled GBS to U937 cells. Ratios of GBS-to-U937 cells were 1000:1, 100:1, and 10:1. Data are represented as the percentage of U937 cells that have GBS attached. The samples were run in triplicate and are represented as the average of three samples.
Figure 2b. Flow cytometric measurement of the attachment of labeled GBS to U937 cells. Ratios of GBS-to-U937 cells were 1000:1, 100:1, and 10:1. Data are represented as the percentage of U937 cells that have GBS attached. The samples were run in triplicate and are represented as the average of three samples.
Figure 3a. Inhibition of GBS attachment to U937 Cells with M18/2. Anti-CD18 (M18/2) antibody was used in an ELISA inhibition assay at various dilutions to determine its effect on GBS attachment to U937 cells. The samples were performed in triplicate and each bar represents the average compared to an isotype control.

$R^2 = .903$
Figure 3b. Inhibition of GBS attachment to U937 Cells with M1/70. Anti-CD11b (M1/70) antibody was used in an ELISA inhibition assay at various dilutions to determine its effect on GBS attachment to U937 cells. The samples were performed in triplicate and each bar represents the average compared to an isotype control.

$R^2 = .814$
Figure 3c. Inhibition of GBS attachment to U937 Cells with BU15. Anti-CD11c (BU15) antibody was used in an ELISA inhibition assay at various dilutions to determine its effect on GBS attachment to U937 cells. The samples were performed in triplicate and each bar represents the average compared to an isotype control.

$R^2 = .550$
there was only minimal inhibition of GBS attachment to U937 cells and the results do not seem to follow a dose-dependent inhibition. It appears that the maximal inhibition with the anti-CD11c antibody was only 20%.

Table 1a shows the results from inhibition studies to determine the effect of anti-CD14 as an inhibitor of GBS adherence to unstimulated U937 cells. Regardless of the concentration of anti-CD14 antibody, it was unable to inhibit binding of GBS to U937 cells compared to the control with no treatment. The mean fluorescence intensity (MFI) of GBS binding increased compared to the control indicating that the anti-CD14 antibody actually increased the number of GBS attached per U937 cell (Table 1b). Table 2a shows the results from inhibition studies using PMA-treated U937 cells. Again, the results show that treatment with anti-CD14 antibody does not inhibit GBS adherence to U937 cells. However, at GBS-to-U937 ratios of 100:1 with a 1/3 dilution of anti-CD14 there was an increase in the number of GBS attached per U937 cell, as well as with a ratio of 1000:1 and a 1/9 dilution of antibody (Table 2b). These results indicate that anti-CD14 does not inhibit GBS adhesion to U937 cells and appears, in some cases, to increase the number of GBS attached to each U937 cell.

The L929 cell line was used as a negative control for flow cytometry because previous work (140) had shown that this cell line poorly bound GBS. Figure 4 shows that there is minimal binding of GBS to L929 cells, eliminating the concern that labeled bacteria were giving false positive results.

**GBS bind to a 60-70 kDa U937 cell surface protein.** Since 100% inhibition was not achieved using the monoclonal antibodies, there may be additional U937 cell proteins interacting with GBS. Figure 5 shows the protein profile of disrupted U937 cells. As expected, there were a number of proteins associated with U937 cells. Figure 6 shows the biotin-labeled surface proteins of U937 cells. Possible receptors on U937 cells for GBS
**TABLE 1**

**EFFECT OF ANTI-CD14 ANTIBODY AS AN INHIBITOR OF GBS ADHERENCE TO UNSTIMULATED U937 CELLS**

**A. PERCENTAGE OF UNSTIMULATED U937 CELLS WITH GBS ATTACHED**

<table>
<thead>
<tr>
<th>GBS CONTROL a</th>
<th>ANTI-CD14 TREATMENT a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNDIL</td>
</tr>
<tr>
<td>1000:1</td>
<td>99.67</td>
</tr>
<tr>
<td>100:1</td>
<td>10.95</td>
</tr>
</tbody>
</table>

a Percentage binding (N=3)

**B. MEAN FLUORESCENCE INTENSITY OF GBS BINDING TO UNSTIMULATED U937 CELLS**

<table>
<thead>
<tr>
<th>GBS CONTROL a</th>
<th>ANTI-CD14 TREATMENT a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNDIL</td>
</tr>
<tr>
<td>1000:1</td>
<td>75.89</td>
</tr>
<tr>
<td>100:1</td>
<td>37.35</td>
</tr>
</tbody>
</table>

a Mean fluorescence intensity (N=3)
b Analysis of variance for all systems indicated a statistically significant difference in the effects of anti-CD14 antibody on bacterial adherence (p=<.001) compared to the control.
### TABLE 2

**EFFECT OF ANTI-CD14 ANTIBODY AS AN INHIBITOR OF GBS ADHERENCE TO PMA-TREATED U937 CELLS**

**A.** **PERCENTAGE OF UNSTIMULATED U937 CELLS WITH GBS ATTACHED**

<table>
<thead>
<tr>
<th>GBS CONTROL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ANTI-CD14 TREATMENT&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RATIO</strong></td>
<td><strong>UNDIL</strong></td>
</tr>
<tr>
<td>1000:1</td>
<td>98.45</td>
</tr>
<tr>
<td>100:1</td>
<td>6.69</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage binding (N=3)

**B.** **MEAN FLUORESCENCE INTENSITY OF GBS BINDING TO UNSTIMULATED U937 CELLS**

<table>
<thead>
<tr>
<th>GBS CONTROL&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ANTI-CD14 TREATMENT&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RATIO</strong></td>
<td><strong>UNDIL</strong></td>
</tr>
<tr>
<td>1000:1</td>
<td>68.30</td>
</tr>
<tr>
<td>100:1</td>
<td>65.90</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean fluorescence intensity (N=3)

<sup>b</sup> Analysis of variance for all systems indicated a statistically significant difference in the effects of anti-CD14 antibody on bacterial adherence (p=<.001) compared to the control.
Figure 4. Attachment of GBS to L929 cells. L929 cells were incubated with various concentrations of GBS-FITC. The samples were analysed by flow cytometry. Each bar represents the average of 3 samples.
Figure 5. **U937 cell proteins separated on SDS-PAGE.**
Lysates of U937 cells were prepared and separated on 12% SDS-PAGE to

determine the protein profiles. Lane 1 contains the molecular mass markers in

kilodaltons, Lane 2 contains 10 μg of U937 cell proteins, Lane 3 contains 20 μg

of U937 cell proteins.
Figure 6. Western Transfer of biotin-labeled U937 cell membrane proteins. U937 cells were biotin-labeled, separated on SDS-PAGE, transferred to nitrocellulose and incubated with streptavidin-HRP. The proteins were visualized using 4-chloro-napthol. Lane 1 contains 10 μg U937 cell proteins, Lane 2 contains 20 μg U937 cell proteins and Lane 3 contains pre-stained molecular mass markers in kilodaltons.
would be expected to be found among these surface proteins.

Figure 7 shows the results from a Western transfer of U937 cell proteins probed directly with GBS. The GBS attached specifically to at least 3 proteins from U937 cells. The first protein has a molecular mass of approximately 60-kDa, the second 55-kDa and the third less than 10-kDa. Lanes 1-3 contain U937 cell preparations that were similarly prepared at different times. Lane 1 does not show the 55-kDa protein detected by GBS, possibly due to technical problems associated with the transfer process from the gel to the nitrocellulose.

A soluble binding assay was performed to ensure that the proteins from U937 cells that the GBS were recognizing were in fact surface proteins. U937 cells were biotin-labeled prior to the lysate preparation and this mixture was used to bind to whole GBS. Figure 8 shows the results from this experiment. When whole GBS were added to the U937 proteins and these bound proteins were separated on SDS-PAGE and transferred to nitrocellulose, 3 proteins were observed. These proteins had molecular masses of approximately 60 kDa, 55 kDa and 20 kDa, comparable to the proteins that were probed with GBS directly. Because the U937 cells were biotin-labeled prior to the addition of the bacteria, the proteins detected by this method were surface U937 cell proteins.

Finally, 2-dimensional electrophoresis was performed to determine the pls of the U937 cell proteins. Figure 9 is a 2-D gel of separated U937 cell proteins stained with Coomassie blue. In a separate assay, these proteins were transferred to nitrocellulose and probed directly with GBS. Figure 10 shows the appearance of one protein at a molecular mass of approximately 55 kDa with a isoelectric point of 5.5 that was directly bound by GBS. This protein is most likely the same 55 kDa protein detected in the soluble binding assay and the Western transfer.
Figure 7. Identification of U937 cells directly bound by GBS. U937 cell proteins were separated on SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was probed directly with GBS. Lanes 1-3 contain U937 cell proteins (prepared the same but at different times). Molecular mass markers in kilodaltons are indicated to the right.
**Figure 8. Soluble Binding Assay.** Western transfer of biotin-labeled U937 cell proteins that were bound by GBS and detected using chemiluminescence. Lane 1 contains molecular mass markers in kilodaltons, Lane 2 contains the eluted U937 cell proteins that were bound by GBS, and Lane 3 contains the GBS control.
Figure 9. 2-Dimensional gel electrophoresis of U937 proteins. U937 cell proteins were separated by 2-D gel. The first dimension used ampholines from 10-3 and the second dimension was a 12% SDS-PAGE. Molecular mass markers in kilodaltons are indicated to the right.
Figure 10. Western transfer of 2-D separated U937 cell proteins probed with GBS. The separated U937 cell proteins were probed directly with GBS and detected using 4-chloro-naphthol. The ampholine range was 5 to 7. Molecular mass markers in kilodaltons are indicated to the right.
DISCUSSION

Opsonic-independent recognition is an important host defense mechanism. This type of recognition may protect nonimmune hosts against infection by bacteria that do not activate the alternative complement pathway or in sites which are poor in opsonins (117). In GBS infection, the individuals most susceptible are those that are immunocompromised or young infants. In this population complement and specific antibody may be decreased. Therefore, there must be opsonic-independent interactions that occur in GBS infection. This study was performed to determine what protein(s) on the surface of phagocytic cells interact with GBS in complement-deficient environment.

U937 cells were chosen as model for GBS adhesion because they are a well established cell line for studying the functions of macrophages (18,67,69). Since it was not known whether GBS could adhere to U937 cells, three separate assays were performed. The visual binding assay originally developed by Glass et al. (49) involves direct microscopic examination of stained cells. This assay is valuable because it offers direct visualization of bacteria binding to macrophages. This assay can distinguish whether a macrophage has one or 20 GBS attached. However, this assay does have shortcomings. It is labor intensive, and lacks reproducibility, and the data are subjectively derived (30).

In addition, only attachment of GBS to PMA-treated U937 can be measured with this assay, since only PMA-treated U937 cells attach to the assay chamber (65,146). GBS indeed bound to PMA-treated U937 cells and multiple washes did not eliminate this binding, indicating that there was a strong adhesion of the bacteria for these cells.

The ELISA assay used to measure bacterial adherence is an adaptation of the ELISA assay originally designed by Ofek, et al. (116), and modified by Sloan and Pistole (140). The ELISA assay has two advantages over the visual binding assay. The first is subjectivity. Because the results were measured by a microplate reader at a particular optical density there is no subjectivity in interpreting the results. The second advantage to
this assay was that attachment of GBS could be measured to both PMA-treated and untreated U937 cells. However, at times this assay can produce a high background that must be taken into consideration when calculating the results. Regardless, the results from this assay supported the results from the visual binding assay.

Flow cytometry provides a reliable means of measuring bacterial attachment to U937 cells. In these studies the U937 cells were in suspension when the bacteria attached. GBS were directly labeled with FITC so there was no need for a secondary reagent. The flow cytometer was able to measure each individual U937 cells as it passed through the laser beam and quantitate the bacteria that were attached. There was no chance of human error in the quantitation of this experiment. The background associated with flow cytometric measurements was minimal so the results were more reliable then the visual binding assay and the ELISA. In addition a control was performed that used L929 cells, a cell line that has been shown to poorly bind GBS (140), to determine if there was labeled bacteria escaping into the laser and thus giving false results. The results from this experiment show that L929 cells were not able to bind GBS with any significance and the results from the flow cytometry experiments did indeed measure adherent bacteria. In any case, the results from all three assay systems showed the ability of U937 cells to bind to GBS. In addition, there was no increase in the number of bacteria bound to stimulated U937 cells compared to unstimulated cells. This suggests that the protein from U937 cells that GBS are adhering to is not upregulated upon PMA treatment.

The β₂-integrins have been implicated in bacterial attachment to phagocytic cells in the absence of exogenous opsonins (117). Using monoclonal antibodies directed against CD18 (the common β-subunit) and CD11b (the α-subunit) there was an decrease in the binding of GBS to U937 cells, suggesting that the β₂ integrins are involved in attachment. This is supported by studies from Antal et al. (7) and from Sloan and Pistolet (141) who show that CD18 and CD11b are partially involved in GBS attachment to macrophages.
The CD11c receptor appeared to be involved in binding but to a much lesser degree than the others. However, 100% inhibition of GBS attachment to U937 cells was not observed using monoclonal antibodies which suggested the involvement of other receptors.

CD14, the LPS-receptor, has been implicated in Gram-positive bacterial attachment to macrophages (86). This receptor was investigated as a potential adhesin for GBS. Results from the soluble binding assay and the Western transfer assay probed with GBS revealed a protein with the same molecular weight as CD14; therefore monoclonal antibody to this receptor was used in inhibition assays to determine if it could block GBS adhesion to U937 cells. Monoclonal antibody to CD14 did not inhibit attachment of GBS to U937 cells and suggests that this receptor is not involved in GBS attachment to macrophages.

Considering the magnitude of receptors on the surface of U937 cells that could potentially be adhesins for GBS, monoclonal antibody inhibition studies did not seem like an appropriate avenue to continue. Instead, studies were undertaken to determine what protein(s) from U937 binds GBS. Using Western blot analysis, it was found that three proteins (60 kDa, 55 kDa and 20 kDa) appeared to be directly involved in bacterial binding to U937 cells. Using two separate assays, one involving whole bacteria to probe separated U937 cell proteins and another involving whole GBS binding to labeled-U937 cell proteins, the same size proteins were observed in each assay and appear to be involved in adherence. These proteins are not in the molecular weight range of the β₂-integrins (144) and are believed to belong to another family of molecules. Stated above CD14 is in the appropriate molecular weight range and was investigated for its role as one of the potential adhesins. The results do not support that it is the receptor involved in bacterial attachment. There are a number of proteins in the molecular weight range of 60-55 kDa that could be potential adhesins. For example, the C1q-receptor (60-kDa) (46) is found on a variety of cells including U937 cells (45). The C1q-receptor has been implicated in the adherence of bacteria to host cells expressing this receptor (46). In order to determine the
true identity of the proteins microsequencing of the proteins is required. Two attempts
have been made at this procedure but there was not enough sequence information to
provide identification of the proteins.

To determine the identities of these molecules, further experimentation needs to be
performed. It is clear that the proteins identified are involved in GBS adherence to U937
cells. The identification of these proteins could provide a means of developing
immunotherapeutic reagents to help fight GBS infection. At the very least, the identity of
these proteins could provide a better understanding of the interactions of bacteria with cells
of the innate immune system, namely macrophages.
CHAPTER II

GROUP B STREPTOCOCCUS ADHERE TO BOVINE POLYMORPHONUCLEAR LEUKOCYTES BY A CD11/CD18- INDEPENDENT MECHANISM

ABSTRACT

The exact mechanism of adherence between phagocytic cells and group B Streptococcus (GBS) is unknown. Because complement and specific antibody may be decreased or absent in infected individuals, adherence mechanisms were studied in the absence of such factors. In the absence of exogenous opsonins, GBS is able to adhere to mammalian phagocytes in a dose-dependent manner. A bovine model of leukocyte adhesion deficiency (BLAD) was used to explore the role of the β₂-integrins and to investigate the involvement of additional receptors in adherence mechanisms of GBS. BLAD individuals are unable to express β₂-integrins on the surface of their leukocytes. Flow cytometry studies indicated that at PMN:GBS ratios of 1:10, 1:100 and 1:1000, 9.55, 18.8 and 76.3%, respectively, of BLAD polymorphonuclear cells (PMNs) had GBS attached. Under comparable conditions 13.8, 32.3 and 95.3% of normal bovine PMNs had GBS attached which is an increase over BLAD, suggesting that β₂ integrins are needed for optimal adherence. However, the mean fluorescence intensity of GBS binding to both the normal and BLAD PMNs was similar at PMN:GBS ratios of 1:10 and 1:100 indicating that the normal and BLAD PMNs bind GBS equally well. Because the β₂-integrins do not seem to be a requisite for GBS adherence, the involvement of other surface proteins from PMNs was investigated. A soluble binding assay showed that GBS binds to a 55-60 kDa protein from the surface of normal, carrier and BLAD PMNs. These data indicate that

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adherence of GBS to PMNs involves multiple receptors. These findings will be useful in developing intervention strategies for human infections due to GBS.

INTRODUCTION

Studies in our laboratory have shown that GBS are able to bind to murine peritoneal macrophages (141) and U937 cells (L.M. Smith, unpublished) in the absence of exogenous complement or antibody. Other investigators have shown that phagocytosis of GBS is dependent on complement receptor type 3 (CR3; CD11b/CD18) present on macrophage and PMN membranes (7,142). Antal et al. (7) showed that anti-CR3 antibody was not able to completely block phagocytosis of GBS by PMNs and macrophages suggesting an additional opsonin-independent recognition/binding site that contributes to phagocytosis. I propose that the first step of phagocytosis, attachment, is independent of CR3 expression on the surface of phagocytes and that additional binding protein(s) exists for GBS. In order to test this hypothesis a bovine adhesion deficiency system was used.

Leukocyte adhesion deficiency (LAD) is an autosomal recessive disease characterized by greatly reduced expression of the heterodimeric β2-integrin adhesion molecules on leukocytes resulting in multiple defects in leukocyte function (6,81). Without β2-integrin expression on their surface, neutrophils are unable to enter the tissues to destroy invading pathogens; consequently, LAD patients suffer recurrent bacterial infections (6,81,127). Molecular cloning of cDNA encoding for part or all of the defective CD18 gene has identified deletions (82,161) or point mutations (8-10,25,161) as well as nonsense mutations and gene deletions (95) in the coding region. These deletions, point mutations and substitutions all affect CD18 cell surface expression. The fact that these substitutions are in highly conserved regions in β2-integrins suggests that these regions are crucial for cell surface expression (8).

The molecular and genetic basis of a granulocytopathy syndrome in Holstein cattle
has been recently identified as a deficiency in the expression of \( \beta_2 \)-integrins due to a point mutation in the codon for the aspartic acid at position 128 of the CD18 proteins (77,139).

This defect in CD18 results in <2% of normal CD18 expression on leukocytes of affected calves. The clinical manifestations of the bovine disease have been extensively studied (76). Bovine leukocyte adhesion deficiency is the only naturally-occurring animal model of CD18 deficiency that is available for research. Because GBS are able to bind to normal bovine PMNs in an antibody- and complement- deficient environment, I used this model to study the role of the \( \beta_2 \)-integrins in the early events of phagocytosis.

**MATERIALS AND METHODS**

**Bacterial strains.** COH31, a type III GBS originally isolated from an infant with meningitis, was provided by Dr. Dennis Kasper (Harvard Medical School) (75). Cultures were stored at -70°C in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) with 10% glycerol.

**Bacterial cell labeling.** A 1 ml volume of overnight stationary phase culture of COH31 in Todd-Hewitt broth was brought to log phase in 10 ml of this medium by incubation at 37°C for 2 h. The cells from the 10 ml culture were washed twice with phosphate-buffered saline (PBS) by centrifugation at 10,000 x g for 10 min. Cells were resuspended in 5-ml of PBS and an equal volume of fluorescein isothiocyanate (FITC) [isomer I] (Calbiochem, La Jolla, Ca.), 5 mg/ml in NaHCO\(_3\) pH 8.0, was added. The suspension was rocked in the dark for 1 h at room temperature. The bacteria were washed three times to remove unbound FITC (10,000 x g for 10 min). After the last wash, bacteria were resuspended in 1 ml of PBS.
**Bovine PMN Isolation.** Bovine PMNs were isolated from the peripheral blood of normal Holstein calves, calves with BLAD (139) and carriers calves that are heterozygous for the CD18 allele (79). The calves were housed at the National Animal Disease Center-USDA, Ames, IA. Whole blood samples were provided by Dr. Marcus Kehrli. PMNs were isolated by centrifuging diluted whole blood (1:3 with PBS) through Histopaque-1077 (10 ml; Sigma, St. Louis, Mo.) at 850 x g for 30 min. The bottom layer containing erythrocytes and PMNs was reserved. Erythrocytes were lysed in cold H₂O after the immediate addition of PBS to restore isotonicity. PMNs were collected by centrifugation at 300 x g for 10 min and resuspended in 10 ml of PBS. Cells were maintained at room temperature and used immediately for membrane preparations or whole cell staining for flow cytometric analysis.

**U937 cells.** U937 cells (ATCC CRL-1593.2) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown as described in Chapter I.

**CD18 Immunostaining.** Whole blood was used for the immunostaining of CD18 on blood leukocytes. Briefly, 100 μl of ACD-anticoagulated blood per animal was placed in 12 x 75 mm polyethylene tubes and monoclonal antibodies were added. Each antibody was an IgG₁ isotype and was directly conjugated with FITC and used undiluted. Following addition of isotype control antibody (10 μl tube⁻¹; clone X-927 from DAKO Corp., Carpinteria, CA), or anti-CD18 antibody (10 μl tube⁻¹; clone MHM23 from DAKO Corp.), the mixtures were left in the dark at room temperature for 15 min. At the end of the incubation period, erythrocytes were lysed by adding 2 mL of cold hypotonic lysing solution (10.56 mM Na₂HPO₄, 2.67 mM NaH₂PO₄, pH 7.34) to each tube for 90 sec, agitating, and isotonicity was restored with 1 ml of cold restoring solution (10.56 mM
NaH$_2$PO$_4$, 0.43 M NaCl, pH 7.25). The tubes were centrifuged again, the supernatant fluid was aspirated, leukocytes pellets were resuspended, and cells were washed twice in 3 ml of PBS. The remaining cellular debris was removed and cells were fixed by resuspending the leukocytes in 0.5 ml FACS® brand lysing buffer (diluted 1:10 in double-distilled H$_2$O; Becton Dickinson, San Jose, CA) for 10 min in the dark at room temperature. Tubes were centrifuged again and the supernatant fluid was aspirated and resuspended in 0.5 ml of sheath fluid (Isoton II, Coulter Diagnostics, Hialeah, FL) for immediate flow cytometric analysis.

**Immunostaining with GBS-FITC.** Aliquots of bovine PMN suspensions containing ~ 1 x 10$^6$ cells were added per well to a 96-well V-bottom plates, followed by the addition of 20-μl of the appropriate GBS-FITC dilution. After incubation at room temperature for 30 min, the cells were washed with PBS by centrifugation at 700g for 2 min and the plates were inverted to remove the fluid. The washed cells were fixed with 200 μl of 0.5% paraformaldehyde in PBS and stored at 4°C in the dark until analyzed by flow cytometry. Prior to analysis, the samples were transferred to 5-ml Falcon tubes (Fisher Scientific, Pittsburgh, PA).

**Flow cytometric analysis of CD18 and GBS attachment.** Adherence of GBS and anti-CD18 antibody to PMNs was measured using the FACScan™ flow cytometer (Becton Dickinson). Data from 10,000 events per sample were acquired. CellQuest™ software (Becton Dickinson) was used for the CD18 fluorescence analysis and Lysis II™ software (Becton Dickinson) was used for the GBS fluorescence analysis. PMNs were gated out from other leukocyte populations based on their forward and side scatter characteristics on dot plots. The FITC fluorescence histograms of cells in this gated region were then plotted, and mean fluorescence intensities (MFI) computed in the geometric
linear mode. Histograms were plotted for each cow that represented background fluorescence and either CD18 fluorescence or GBS fluorescence. Statistical significance was determined by Students' t-test using Statview™ software. Linear regression was determined for binding and MFI studies using the Statview™ software.

Identification of bovine PMN surface proteins. Bovine PMNs, isolated as described above, were washed extensively with PBS, reconstituted in 1 ml of PBS, and 100 μg/ml of biotin (Pierce, Rockland, IL) dissolved in dimethylsulfoxide (DMSO) was added to each cell preparation. The cells were incubated for 1 h at room temperature in the dark with gentle rocking. The washed cells were reconstituted in 1 ml of 1% Triton X-100, 0.1% SDS in PBS and centrifuged (12,000 x g) to remove nuclear debris. Equal volumes (10-μl) of the membrane preparation and Laemmli sample buffer (87) were mixed and the proteins separated on a 12% SDS-PAGE gel. The proteins were transferred to nitrocellulose (150) and the blotted proteins incubated in 5% skim milk buffer (Difco) for 1 h. The blot was then incubated at room temperature for 1 h with avidin-horseradish peroxidase (HRP) (Pierce) at a dilution of 1:1000 in 1% skim milk buffer in PBS. The blot was washed 4 times in PBS and the biotinylated proteins were visualized using chemiluminescence (Amersham, Arlington Heights, IL).

Identification of bovine PMN proteins involved in GBS adhesion.
Todd-Hewitt Broth (10 ml) was inoculated with 1 ml of an overnight culture of GBS and the culture incubated at 37°C for 2 h. The cells were washed as previously described and the bacterial pellet was reconstituted in 5% gelatin in PBS and incubated at 37°C for 2 h. Concurrently, 200-μl of biotinylated membrane preparations from the bovine PMNs were added to 800-μl of 5% gelatin, 1% Triton X-100, 0.1 % SDS in PBS and incubated at 37°C for 2 h. Experimental systems, containing GBS and bovine neutrophil membranes,
and control systems, containing GBS and buffer, were incubated for 4 h at 37°C with end-over-end rocking. The bound bovine surface proteins were eluted from washed bacteria with 50-μl Laemmli sample buffer and boiled for 1 min. The GBS were centrifuged at 12,000g and the supernatant fluid containing the eluted proteins was retained. Ten microliter samples of the isolated protein suspension and of the control were separated on 12% SDS-PAGE (mini-gel apparatus, Biorad™). Proteins were transferred to nitrocellulose, blocked and incubated with avidin-HRP as described above. Isolated bovine PMN proteins that bind to GBS were visualized with chemiluminescence.

**Anti-CD14 antibody preparation.** Anti-CD14 antibody (60bca) was prepared as described in Chapter I. The antibody was used to stain the surface of bovine PMNs to determine how much CD14 the cells express. The staining was performed as described above for anti-CD18. In addition, inhibition assays were performed using anti-CD14. The experiments were performed as described in Chapter I.

**RESULTS**

**Immunofluorescent analysis of bovine PMNs with anti-CD18 monoclonal antibody.**

Normal (#426), carrier (#1366) and BLAD (#1365 and #1367) PMNs were incubated with anti-CD18 monoclonal antibody to determine the degree of CD18 cell surface expression. Figure 1 shows that PMNs from normal and carrier animals express comparable levels of CD18 on their cell surface as evidenced from the peak shift compared to an isotype negative control. For these animals ~100% of the PMNs were positive for CD18. The carrier animal expresses between 65-75% surface CD18 as compared to the normal and isotype controls (M. Kehrli, personal communication). Compared to the
Figure 1. CD18 expression on bovine PMNs. CD18 expression on bovine PMNs was measured using flow cytometry. Calf 1366 is the carrier animal, cow 426 is the normal animal and calves 1365 and 1367 are the BLAD animals.
isotype control, BLAD animals (#1367 and #1365) have only minimal cell surface expression of CD18.

**Immunofluorescent analysis of bovine PMNs with GBS.**

FITC-labeled GBS at bacteria-to-PMN ratios of 1000:1, 100:1 and 10:1 were incubated with bovine PMNs to determine the percentage of PMNs with GBS attached as well as the mean fluorescence intensity of binding. PMNs were also incubated with (PBS) as a control to determine the background autofluorescence. Each sample was performed in triplicate. Figure 2 shows that GBS binds to normal, carrier and BLAD PMNs in a dose-dependent manner and both the percentage of cells positive for GBS binding (as indicated by a peak beyond 10^1) and the mean fluorescence intensity of binding decrease as the bacteria-to-PMN ratio approaches 10 compared to the PBS control. Each sample was run in triplicate and the data presented in Fig. 2 are a representative pattern for the normal, carrier and BLAD animals examined.

Figure 3a is a graphic depiction from one experiment showing the average percentage of PMNs with GBS attached from three samples. PMNs from the normal animal at GBS:PMN ratios of 1000:1 had 95.3% PMNs bound by GBS, which decreases to 32.3% and 13.8% at ratios of 100:1 and 10:1 respectively. The carrier animal had 80.4%, 22.4% and 12.8% PMNs bound by GBS for the same GBS:PMN ratios. For the BLAD 2 animal (#1365), 76.3%, 18.8% and 9.6% of the PMNs had GBS attached. The lowest binding was observed by BLAD 1 animal (#1367). At the stated GBS:PMN ratios 53.4%, 9.1% and 4.3% of the PMNs had GBS attached. At GBS:PMN ratios of 1000:1 and 100:1 there were significantly more PMNs with GBS attached to the normal animal compared to the BLAD animals, suggesting optimal binding may be CD18 dependent.

The experiment was repeated to include a larger population size. In this study the ability of GBS to bind 3 additional normal and carrier animals and one BLAD animal was
Figure 2. Attachment of GBS-FITC to bovine PMNs. Bovine PMNs were incubated with GBS-FITC at PMN-to-GBS ratios of 1:1000 (B), 1:100 (C), and 1:10 (D). PBS was used as a negative control (A) to determine background autofluorescence. One representative experiment is shown out of three.
Figure 3a. Percentage of Bovine PMNs that have GBS attached. Bovine PMNs were incubated with GBS-FITC at GBS-to-PMN ratios of 1000:1, 100:1, and 10:1. The data represented are from experiment one. There were one normal, one carrier and two BLAD animals used for this experiment. Each bar represents the average from 3 samples. * Indicates statistical difference (p>0.001) between normal and BLAD animals.
examined. These data are represented graphically in figure 3b and support our initial findings. GBS bound to PMNs from all samples in a dose-dependent manner and a larger number of PMNs from normal and carrier animals had adherent GBS attached. These findings indicate that bacterial attachment occurs regardless of whether the PMNs are from BLAD, carrier or normal cows. The findings from this experiment support the initial findings with the smaller sample population.

These same cell samples were examined for mean fluorescence intensity, which measures the amount of fluorescence per cell and, hence, indicates the extent of GBS attachment to these PMNs. As shown in fig. 4a, based on the four animals in our original sample, there were no consistent differences among the normal, carrier and BLAD samples. In the expanded study, summarized in fig. 4b, these results are confirmed. Thus, for the PMNs that bound GBS, cells from normal, carrier, and BLAD animals were comparably effective.

**Identification of surface proteins on PMN preparations.**

Because GBS were able to bind to PMNs from BLAD animals that only express minimal amounts of CD18 on their cell surface, β2-integrin expression is not an essential requisite for attachment. This suggested that additional receptors from PMNs may be involved in GBS attachment. To determine if another protein/receptor on the surface of PMNs facilitates GBS binding, crude membrane preparations of the PMNs from each of the animals were prepared. Figure 5 depicts a Western blot of separated PMN proteins that had been biotin labeled prior to isolating the membrane fraction. Biotin will only bind to surface expressed proteins and will not traverse the membrane (92). If additional proteins are involved in GBS attachment they would be surface proteins. The proteins were also separated on 12% SDS-PAGE and stained with coomassie blue to visualize all proteins (Fig. 6). In addition to normal, carrier and BLAD cell lysates, lysates from U937 cells
Figure 3b. Percentage of Bovine PMNs that have GBS attached.
Bovine PMNs were incubated with GBS-FITC at GBS-to-PMN ratios of 1000:1, 100:1 and 10:1. The data presented are from experiment two. There were three normal, three carrier and one BLAD animals used in this experiment. For the normal and the carrier animals each bar represents the average from three animals in triplicate and standard deviation bars are represented. The BLAD bar represents the average of three samples.
Figure 4a. Mean fluorescence intensity of GBS bound to bovine PMNs. Bovine PMNs were incubated with GBS-FITC at GBS-to-PMN ratios of 1000:1, 100:1, and 10:1. The data presented are from experiment one. One normal, one carrier, and two BLAD animals were used in this experiment. Each bar represents the average from 3 samples.
Figure 4b. Mean fluorescence intensity of GBS bound to bovine PMNs. Bovine PMNs were incubated with GBS-FTTC at GBS-to-PMN ratios of 1000:1, 100:1, and 10:1. The data presented are from experiment two. Three normal, three carrier, and one BLAD were used for this experiment. For the normal and carrier animals each bar represents the average from three animals in triplicate and standard deviation bars are shown. The BLAD bar represents the average of three samples.
Figure 5. Identification of surface proteins from bovine PMNs.
Cells were labeled with biotin prior to membrane lysate preparations. The labeled proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose. Following incubation with avidin-HRP, proteins were visualized using chemiluminescence. Lane 1 contains proteins from a carrier animal, lanes 2 and 4 contain BLAD proteins, and lane 3 contains proteins from a normal animal. Molecular weight markers are indicated.
Figure 6. Coomassie blue stained proteins from U937 cells and bovine PMNs. Lysates from U937 cells and bovine PMNs were prepared and separated on 12% SDS-PAGE. The proteins were visualized with coomassie blue stain. Lane 1 contains U937 cell proteins, lane 2 is empty, lane 3 contains proteins from a carrier animal, lanes 4 and 6 proteins from BLAD, lane 5 proteins from a normal animal and lane 7 contains the molecular mass markers in kilodaltons.
were also run in this experiment. Figure 6 shows the protein profiles from the separated proteins.

Because of the magnitude of surface associated proteins from PMNs, a soluble binding assay was performed to identify the binding protein. Since the PMNs had been biotin-labeled prior to membrane lysate preparation only the surface proteins would be detected. A 55-60 kDa protein was detected when whole GBS was used to bind PMN lysates (Fig. 7). A control, consisting of GBS with no added PMN lysate, was included. In addition, a soluble binding assay was also performed using U937 cells and GBS to determine if the same molecular weight protein was seen when compared with the bovine samples. Previous studies indicated that GBS bound to a 60-55 kDa from the surface of U937 cells (Chapter I). When the proteins from the bovine samples and U937 samples were analyzed together a similar molecular weight protein is observed. Because this protein was present on the surface of normal, carrier and BLAD PMNs (and perhaps the surface of U937 cells), I speculate that this protein is involved in the initial attachment of GBS to PMNs, even in the absence of $\beta_2$-integrin expression.

**CD14 involvement.** Based on the molecular weight of the identified protein inhibition studies using anti-CD14 antibody were performed. CD14 is in the same molecular weight range of the detected protein. PMNs from normal, carrier, and BLAD animals were examined for their ability to express CD14. Figure 8 shows that all animals regardless of CD18 expression also express CD14. This antibody was also used in an inhibition assay to determine if it could inhibit GBS attachment to bovine PMNs. As with U937 cells, this antibody had no effect on the attachment of GBS to bovine PMNs (Table 1).
Figure 7. Soluble binding assay. Surface proteins from U937 cells and bovine PMNs were labeled with biotin prior to lysate preparations. These preparations were incubated with whole GBS, allowed to bind and unbound proteins removed. The proteins that bound directly to GBS were eluted and separated on SDS-PAGE. They were transferred to nitrocellulose and the proteins that bound GBS directly were visualized using chemiluminescence. Lane 1 contains proteins from U937 cells, lane 2 contains proteins from carrier PMNs, lanes 3 and 5 contain BLAD proteins and lane 4 contains proteins from normal PMNs, lane 6 is empty, lane 7 contains a GBS control, and lane 8 contains prestained molecular mass markers in kilodaltons.
Figure 8. Percentage of bovine PMNs that express CD14. Bovine PMNs were stained with 60bca (anti-CD14) antibody conjugated to biotin. The antibody was detected using avidin conjugated to phycoerythrin. The samples were analyzed using flow cytometry. Each bar represents the average of 2 samples compared to an isotype control.
TABLE 1
EFFECT OF ANTI-CD14 ANTIBODY AS AN INHIBITOR OF GBS ADHERENCE TO BOVINE POLYMORPHONUCLEAR LEUKOCYTES

PERCENTAGE OF BOVINE PMNS WITH GBS ATTACHED*

<table>
<thead>
<tr>
<th>Ratio</th>
<th>GBS Control</th>
<th>Anti-CD14 Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000:1</td>
<td>93.82</td>
<td>93.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100:1</td>
<td>33.97</td>
<td>28.73&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Results are from one representative bovine with normal CD18 surface expression.
<sup>a</sup> Percentage binding (N=3)
<sup>b</sup> Analysis of variance for all systems indicated no statistically significant difference in the effects of anti-CD14 antibody on bacterial adherence (p=<.5)

PERCENTAGE OF BLAD PMNS WITH GBS ATTACHED<sup>0</sup>

<table>
<thead>
<tr>
<th>Ratio</th>
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<tr>
<td>1000:1</td>
<td>44.95</td>
<td>39.83&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>100:1</td>
<td>7.08</td>
<td>7.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

<sup>0</sup>Results are from one representative bovine with BLAD.
<sup>a</sup> Percentage binding (N=3)
<sup>b</sup> Analysis of variance for all systems indicated no statistically significant difference in the effects of anti-CD14 antibody on bacterial adherence (p=<.5)
DISCUSSION

Opsonic-dependent recognition and phagocytosis of bacteria by phagocytic cells has been well established. Many organisms such as *Listeria monocytogenes* (30), *Mycobacterium leprae* (134,135), *Legionella pneumophila* (119), and *Leishmania* spp.(126) undergo complement-mediated binding and uptake by macrophages via complement receptors (CR1, CR3 or CR4). There is, however, a large body of evidence showing that binding and phagocytosis can take place in the absence of opsonins, suggesting that organisms may rely on alternative mechanisms for recognition of invading pathogens (117). *Bordetella pertussis* (61), *E. coli* (43), some strains of *Mycobacterium tuberculosis* (145) and GBS (7) are recognized by phagocytic cells and are phagocytosed in environments devoid of complement. The exact mechanism of non-opsonic recognition is still under debate.

The goal of this study was to determine the role of the β₂-integrins in bacterial recognition under opsonin-deficient conditions and to uncover any additional proteins that may be involved. The β₂-integrin family is a group of structurally and functionally related glycoproteins present on the surface of leukocytes (144). Antal *et al.* reported that CD11b/CD18 expression on the surface of phagocytes was critical for phagocytosis of GBS and blocking these proteins with monoclonal antibody led to decreased phagocytosis (7), indicating the involvement of other receptors. Graham *et al.* showed that inhibition of target binding is not required for inhibition of phagocytosis by anti-CD11b/CD18, suggesting that the anti-phagocytic effect of these antibodies is exerted at some step in the ingestion process after the adherence necessary for target binding has been engaged (54). An additional investigation by Gresham *et al.* showed that phagocytosis by unstimulated PMN is both CD11b/CD18-independent and -dependent and that phagocytic uptake is dependent on multiple proinflammatory signals on PMNs (56). They showed that CD11b/CD18-dependent mechanisms of ingestion also require the activation of protein

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kinase C. Members of our laboratory and others (117) speculate that the adhesion is a multi-step process that ultimately leads to internalization of the attached bacteria.

To determine the role of β₂-integrin involvement during complement-independent binding, bovine PMNs from calves with BLAD were studied and compared with normal bovine PMNs. This model has been used by Rosenthal et al. to study Leishmania major macrophage interactions (126). GBS was able to bind to normal, carrier, and BLAD PMNs under complement-deficient conditions. The binding of GBS to the normal and carrier animals was greater than that of BLAD indicating that CD18 expression may provide maximum adhesion. However, since there was significant binding to the BLAD PMNs, it was concluded that CD18 expression is not essential for binding. This is supported by a study by Goodrum et al. showing that the nitric oxide (NO) response of macrophages to GBS appears to be mediated through direct or indirect interaction of GBS with CR3 in a manner that triggers phagocytosis (52). Phagocytosis and killing of GBS appear to be CD18-dependent (7,52); however direct binding of GBS to CR3 is still unproven. This study indicates that the binding of GBS to BLAD PMNs is CD18-independent and suggests the involvement of other surface receptors.

The existence of a GBS-binding protein on normal and BLAD PMNs was investigated on the assumption that CD18-independent recognition in each population is mediated by the same protein. GBS were used to elute the binding protein from PMNs, yielding a molecule with the calculated molecular mass of 55-60 kDa. This protein was present on PMN samples from normal, carrier and BLAD suggesting that this protein is involved in the attachment of GBS to PMNs. A protein of the same molecular weight was found on the surface of U937 cells (Chapter I) which suggests a similar binding mechanism in these two cell types.

CD14 is a molecule found on the surface of macrophages and PMNs and is one of the receptors for LPS. Monoclonal antibody to this molecule has been used to show that
bovine alveolar macrophages express CD14 (80). Because the molecular weight of the identified GBS binding protein and CD14 are similar, inhibition studies were performed to determine if the identity of the binding protein was CD14. In inhibition studies this antibody was not able to block attachment of GBS to bovine PMNs, indicating that CD14 is not the binding protein. This is supported by the studies with U937 cells (Chapter I).

Further studies are required to determine if this protein is the first in a series that must be engaged to achieve optimal binding. Does this protein need to be engaged to get internalization of GBS or is it simply a binding protein? Is this protein present on the surface of PMNs from other species? These are questions that remain unanswered; however, we have described the existence of this protein and shown its role in the adhesion process of GBS to PMNs regardless of CD18 expression. This study also showed that a similar molecular weight protein exists on the surface of U937 cells. If these proteins are the same, this suggests evolutionary conservation of the protein and further supports its role in host defense mechanisms against bacteria.
CHAPTER III

LISTERIA MONOCYTOGENES AND GROUP B STREPTOCOCCI USE THE SAME RECEPTORS FOR ADHESION TO MACROPHAGES

ABSTRACT

Listeria monocytogenes and group B streptococci are pathogenic organisms that cause similar diseases in neonates. These organisms are also similar at the genetic level. The mechanisms of bacterial attachment of these organisms to macrophages are not completely understood. This study was undertaken to determine if these organisms adhere to the same receptors on macrophages. Listeria was able to bind to phorbol myristate acetate-treated and -untreated U937 cells in a dose-dependent manner. Complement receptor type 3 was not the primary receptor for attachment of Listeria to polymorphonuclear leukocytes as measured by flow cytometry. Previous studies have shown similar results for GBS. Competition ELISA assays showed that Listeria could inhibit attachment of GBS to U937 cells in a dose-dependent manner and the same was found in the reverse situation. Western blot analysis revealed that Listeria and GBS bind to 3 proteins from U937 cells with molecular masses of 60, 55 and 20 kDa. These results indicate that GBS and Listeria use the same receptors for adhesion to macrophages.

INTRODUCTION

Microbial adhesion is an important stage in the successful colonization and dissemination of microorganisms in the host (15). In addition, bacterial adherence to host immune cells, such as macrophages, is an important step in the clearance of
microorganisms from the host's body (105). The mechanisms that mediate bacterial adherence to host cells are not clearly understood and can vary from organism to organism. This study was undertaken to determine if the same receptors on phagocytic cells recognize and bind two different microorganisms, group B *Streptococcus* and *Listeria monocytogenes*.

*L. monocytogenes* is a facultatively anaerobic, nonsporeforming, Gram-positive rod that grows in the temperature range of -0.4°C to 50°C. This organism is catalase-positive and oxidase-negative and expresses a β-hemolysin, which produces zones of clearing on blood agar. The organism possesses peritrichous flagella that are responsible for the characteristic tumbling motility seen when the organism is cultured at 20 to 25°C (35). Listeria is widely present and has been isolated in soil, plants, surface water samples, sewage, slaughterhouse waste, milk of normal and mastitic cows and in human and animal feces. This organism has been isolated from domestic animals including cattle, sheep, goats and poultry, but it is infrequently isolated from wild animals (35).

The first confirmed isolations of the bacteria from infected individuals, following initial description, were made in 1929 by Gill from sheep and by Nyfieldt from humans (15). Since that time, sporadic cases of listeriosis have been reported, often in workers in contact with diseased animals or contaminated food. Epidemiologic investigation of several outbreaks of listeriosis during the 1980s demonstrated that epidemic listeriosis is a foodborne disease (137).

This organisms usually causes disease in individuals with certain underlying conditions such as pregnancy, neoplastic disease, diabetes mellitus, alcoholism, cardiovascular disease and AIDS (35). These conditions cause suppression of the immune system and increase the chances of listeriosis. In nonpregnant adults, the clinical symptoms of listerial infection includes central nervous system infections and primary bacteremia. Both endocarditis and meningitis are common manifestations of listeriosis in
adults (35). Listeriosis may develop at any time during pregnancy, although most infections are detected in the third trimester. Pregnant women infected with *L. monocytogenes* may experience only a mild flu-like illness, with fever, headache, and occasionally gastrointestinal symptoms. Intrauterine infections most likely result from transplacental transmission following maternal bacteremia, although some intrauterine infection may be the result of ascending spread of vaginal colonization (137). Intrauterine infection can cause amnionitis, preterm labor, spontaneous abortion, still birth, or early-onset infection of the neonate.

As with group B streptococcal infection, there are two clinical forms of listeriosis in infants, early- and late-onset disease syndromes. Early-onset listeriosis results from intrauterine infections, which can cause clinical illness in the newborn at birth or shortly thereafter (137). The clinical manifestations of disease are most often sepsis, although granulomatosis infanitisepticum, an overwhelming form of infection, occurs less frequently (137). Early-onset disease may be associated with aspiration of infected amniotic fluid, which can lead to respiratory distress.

Late-onset listerial infection occurs several days to weeks after birth. Infants are usually full term, healthy at birth, and delivered to mothers who have had uncomplicated pregnancies (137). Late-onset listeriosis, like late-onset GBS disease, is more likely to present as meningitis (137). Evidence of central nervous system infection was present in 93% of late-onset cases reported between 1967 and 1985 in Britain (100). Fatality rates appear to be lower in late-onset compared to early-onset disease cases (137).

The highest incidence rate of listerial infection is usually seen in neonates, followed by those older than 60 years, the proportion of cases not associated with pregnancy appears to be on the increase (35). Mortality rates calculated worldwide, using data obtained from a 1989 survey, ranged from 13 to 34% and it has been suggested that listeriosis may well be the leading fatal food-borne infection in the United States (44).
Studies with *Listeria* have focused on how this organism is able to evade the host's immune system. Once *Listeria* enters the macrophage it can readily lyse the phagosome allowing the organism to replicate inside the cytoplasm (106,149). The exact mechanisms that mediate binding and phagocytosis of *Listeria* are not entirely understood. Drevets and Campbell (28) have shown that listericidal macrophages use CR3 as the major phagocytic receptor to bind and internalize *Listeria*. In contrast, in nonlistericidal macrophages CR3 plays only a small role in attachment and phagocytosis of *Listeria* (29). Drevets and Campbell went on to further show that CR3-mediated phagocytosis of *Listeria* is required for macrophages to kill this organism and that phagocytosis through other receptors leads to permissive growth.

It has been shown that CR3 may be involved in the attachment and phagocytosis of GBS to macrophages (7,141); however, I have shown using bovine LAD PMNs that CR3 is not required for attachment of GBS (Chapter II). I have also shown that there is a protein of similar molecular weight on both U937 cells and bovine PMNs that bind GBS and is not a member of the β₂-integrin family based on molecular weight. This study was undertaken to determine if *Listeria*, an organism that is similar at the clinical (35,137) as well as the genetic level (23) to GBS, is able to adhere to bovine PMNs and U937 cells by the same molecular weight protein(s) as GBS.

**MATERIAL AND METHODS**

**Bacterial Strains.** GBS type III, designated COH31, was provided by Dr. Dennis Kasper, Channing Laboratory (Harvard Medical School). This strain was originally isolated from infants with meningitis (74). It was grown and maintained in our laboratory as previously described (Chapter I).
A clinical isolate of *Listeria monocytogenes* (122) was provided by Dr. Frank Rodgers, University of New Hampshire. The organism was maintained as a frozen stock in Trypticase soy broth (T-soy) (Difco Laboratories, Detroit, MI) with 10% dimethyl sulfoxide (DMSO) at -70°C. For bacterial studies, the microorganism was thawed and streaked onto T-soy agar plates and single colonies were inoculated into T-soy broth and incubated overnight at 37°C.

**Cell lines.** U937 cells (146) were obtained from the American Tissue Culture Collection (ATCC) and were maintained and grown in our laboratory as described in Chapter I.

**Bacterial labeling with biotin and fluorescein isothiocyanate (FITC).**

*Listeria* were grown at 37°C overnight (18 h) in T-soy broth. The bacteria were washed extensively (3 X) in phosphate-buffered saline (PBS) and pelleted by centrifugation (10,000 x g). At this point the bacteria were either labeled with biotin for competition studies or with FITC for flow cytometry studies. GBS was labeled with biotin as described in Chapter III.

Washed *Listeria* were labeled with biotin (Pierce, Rockford, IL) at 0.1 mg/ml for 1 h at room temperature, in the dark, with gentle rocking. Biotin was removed with 4 PBS washes. For competition studies the bacteria were labeled fresh each time.

Washed *Listeria* were labeled with FITC (1 mg/ml) for 1 h at room temperature, in the dark, with gentle rocking. The bacteria were washed 4 x with PBS to remove unbound FITC. Bacteria were labeled fresh for each flow cytometry experiment.

**Isolation of Bovine PMNs.** Bovine blood samples from normal, carrier (79) and BLAD (139) cows were provided by Dr. Marcus Kehrli (National Animal Disease Center-USDA, Ames, IA). The PMNs were isolated as described in Chapter II.
Biotin-labeling and cell lysate preparations of U937 cells. U937 cells were labeled with biotin for soluble binding assays. Biotin (0.1 mg/ml) was added to the cells, which were incubated for 1 h at room temperature, in the dark, with gentle rocking. The cells were washed 4 x with PBS to remove the unbound biotin. After the final wash, 1 ml of 0.1% sodium dodecyl sulfate (SDS) and 1.0% Triton X-100 in PBS was added to pelleted cells. The cell mixture was stored at -70°C until needed for the soluble binding assay.

Immunostaining with Listeria-FITC. Aliquots of bovine PMN suspensions, untreated U937 cells and PMA-treated U937 cells (Chapter I) containing ~ 1 x 10^6 cells were added per well to a 96-well V-bottom plates, followed by the addition of 20-μl of the appropriate Listeria-FITC dilution. After incubation at room temperature for 30 min, the cells were washed with PBS and centrifuged at 700g for 2 min and the plates were inverted to remove the fluid. The washed cells were fixed with 200 μl of 0.5% paraformaldehyde in PBS and stored at 4°C in the dark until analyzed by flow cytometry. Prior to analysis, the samples were transferred to 5-ml Falcon tubes (Fisher Scientific, Pittsburgh, PA).

Flow cytometric analysis Listeria attachment. Adherence of Listeria to bovine PMNs and U937 cells was measured using the FACScan™ flow cytometer (Becton Dickinson). Data from 10,000 events per sample were acquired. Lysis II™ software (Becton Dickinson) was used for the Listeria fluorescence analysis. PMNs were gated out from other leukocyte populations based on their forward and side scatter characteristics on dot plots. The FITC fluorescence histograms of cells in this gated region were then plotted, and mean fluorescence intensities (MFI) computed in the geometric linear mode. Histograms were plotted for each cow and U937 preparation that represented background fluorescence and Listeria fluorescence. Further analysis was performed using Statview™.
software. Linear regression was calculated for the competition studies.

**Competition Studies with GBS and *Listeria.*** For the enzyme-linked immunosorbent assay (ELISA) assay both PMA-treated U937 cells and untreated U937 cells (1 x 10^5 cells/well) were plated into separate 96-well ELISA plates (Immunolon; Dynatech, Chantilly, VA). The cells were dried overnight at room temperature. The microtiter wells were blocked with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Unlabeled-bacteria (either GBS or *Listeria*), at various concentrations, were added to the assay wells containing U937 cells and the plates were incubated for 2 h at room temperature. The unbound bacteria were removed with 3 washes of PBS. Biotin-labeled bacteria were added at a constant concentration to the wells with the unlabeled bacteria (*i.e.*, if unlabeled *Listeria* was added at various concentrations then labeled GBS would be added to the wells at a constant concentration). A control well of labeled bacteria and U937 cells was performed to determine maximum binding. Following a 2-h incubation at room temperature, the unbound bacteria were removed with 3 PBS washes. The bound, labeled bacteria were detected with streptavidin (SA) conjugated to horseradish peroxidase (HRP) (1:2500) (Pierce) in 1% BSA/PBS, with a 2 h incubation. The SA-HRP was removed with 3 washes of PBS. Fifty microliters of the substrate, 2,2’ azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS)(Sigma), plus 1% hydrogen peroxide (H₂O₂) was added to each well and incubated for 15-30 min. Bacterial attachment was measured using a microplate reader (Bio-Rad, Hercules, CA) at OD₄₀⁵ₙₘ. Inhibition was calculated as: 100 – (OD₄₀⁵ₙₘ test/OD₄₀⁵ₙₘ control x 100) = % inhibition

**Identification of U937 cell proteins involved in GBS and *Listeria* adhesion.** GBS and *Listeria* were freshly grown for each soluble binding assay. The washed bacterial pellets were reconstituted in 5% gelatin in PBS and incubated at 37°C for 2 h.

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Concurrently, 200-μl of biotinylated cell lysates from the U937 cells were added to 800-μl of 5% gelatin, 1% Triton X-100, 0.1 % SDS in PBS and incubated at 37°C for 2 h. Experimental systems, containing GBS and U937 cells or *Listeria* and U937 cells, and control systems, containing GBS and buffer or *Listeria* and buffer, were incubated for 4 h at 37°C with end-over-end rocking. The bound U937 cells proteins were eluted from washed bacteria with 50-μl Laemmli sample buffer and boiled for 1 min. The bacteria were centrifuged at 12,000 x g and the supernatant fluid containing the eluted proteins were retained. Ten microliters of the isolated protein suspension and of the control were separated on 12% SDS-PAGE (mini-gel apparatus from Biorad™). Proteins were transferred to nitrocellulose, blocked and incubated with avidin-HRP as previously described in Chapter 1. The isolated U937 cells proteins that bound to GBS and *Listeria* were visualized with chemiluminescence.

**RESULTS**

*Listeria bind U937 cells.* *Listeria* were able to adhere to PMA-treated and untreated U937 cells as measured by flow cytometry (Fig. 1a). The percentage of U937 cells, regardless of PMA treatment, that had *Listeria* attached decreased in a dose-dependent manner. In addition, the number of *Listeria* per U937 cell also decreased as the number of bacteria decreased (Fig. 1b). Treatment of U937 cells with PMA did not affect the number of bacteria attached, suggesting that the receptor for *Listeria* is not upregulated with PMA treatment.

**Involvement of CR3 in *Listeria* attachment.** To determine the role of CR3 in the attachment process of *Listeria* I used a bovine LAD model (Chapter II). The ability of
Figure 1a. *L. monocytogenes* bind to U937 cells. *Listeria* at ratios of 1000:1, 100:1 and 10:1 were incubated with U937 cells (unstimulated or PMA-treated) and the percentage of U937 cells with *Listeria* attached was determined using flow cytometry. Each bar is the average of three samples.
Figure 1b. Mean fluorescence intensity of *Listeria* binding to U937 cells. Attachment of *Listeria* to U937 cells was measured using flow cytometry. At various bacteria-to-U937 cell ratios (1000:1, 100:1, and 10:1) the number of *Listeria* attached per U937 cells was measured. Each bar represents the average of 3 samples.
Listeria to attach to bovine PMNs expressing normal levels of CR3, slightly decreased levels of CR3 and no CR3 was measured using flow cytometry. Figure 2a shows that Listeria was able to bind to bovine PMNs regardless of CR3 expression. The binding occurred in a dose-dependent manner. Table 1 shows that at ratios of 1000:1 and 100:1 (Listeria-to-PMNs) there was a significant difference in the ability of BLAD PMNs to bind Listeria compared to the normal controls. It appears that for optimal binding to occur CR3 expression is necessary.

Figure 2b shows that the number of Listeria attached to each PMN also follows a dose-dependent decrease as the number of bacteria are decreased. Even though there was a lower percentage of BLAD PMNs with Listeria attached, the number of bacteria per cell was comparable to or greater than that of the normal PMNs. Regardless of CR3 expression, Listeria was able to attach to bovine PMNs indicating that CR3 is not an essential requisite for attachment.

**Competition for binding to U937 cells with GBS and Listeria.** A competition ELISA was performed to test the ability of one organism to block the attachment of another to U937 cells. If GBS and Listeria use the same receptors on U937 cells to attach then they should be able to compete for binding. Figure 3a shows the results of a competition assay in which Listeria was bound to U937 cells prior to the addition of GBS. The presence of Listeria on U937 cells was able to inhibit the attachment of GBS. The R² value of .949 indicates that the dilution of Listeria was directly responsible for the inhibition effect observed in this assay. Figure 3b shows the opposite experiment with GBS attached to the U937 cells prior to the addition of Listeria. GBS was able to block the attachment of Listeria to U937 cells in a dose-dependent manner. An inhibition of almost 60% was observed at the lowest dilution of GBS.
Figure 2a. Percentage of Bovine PMNs with *Listeria* attached.

At *Listeria*-to-PMN ratios of 1000:1, 100:1 and 10:1 bacterial attachment to bovine PMNs was measured using flow cytometry. Each bar represents the average of 3 samples.
Table 1

Analysis of the percentage of PMNs that have *L. monocytogenes* attached to determine if CD18 expression effects bacterial attachment.

<table>
<thead>
<tr>
<th>Bovine Sample unpaired comparison (1000:1)</th>
<th>Mean Difference</th>
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<sup>a</sup> Based on analysis of variance. p Values based on comparisons of the percentage of *Listeria* bound between normal and carrier cows, normal and normal cows, BLAD and carrier cows and normal and BLAD cows. This is based on CD18 expression by the various cows. There was a significant difference (*p* < 0.01) in attachment between the normal cows and the BLAD cows at the ratios of 1000:1 and 100:1 (*Listeria*-to-PMNs) suggesting a possible role for CD18 as an adhesion for *Listeria*. Normal 1 is 1608 and normal 2 is 1306.
Figure 2b. Mean fluorescent intensity of *Listeria* attachment to bovine PMNs. The number of *Listeria* attached to bovine PMNs was determined by flow cytometry. At bacteria-to-PMN ratios of 1000:1, 100:1 and 10:1 the number of bacteria per PMN was measured. Each bar represents the average of three samples.
Figure 3a. **Competition Assay: GBS constant**. *Listeria* was added at 5-fold dilutions to U937 cells and allowed to adhere. GBS-biotin, at a constant concentration, was added to determine if it could displace the bound *Listeria*. The data are expressed as percent inhibition, based on the number of GBS that were unable to bind due to listerial attachment.

$R^2 = .949$
Figure 3b. Competition Assay: *Listeria* constant. GBS was added at 5-fold dilutions to U937 cells and allowed to adhere *Listeria*-biotin, at a constant concentration was added to determine if it could displace the bound GBS. The data are expressed as percent inhibition, based on the number of *Listeria* that were unable to bind due to GBS attachment.

R² = .931
GBS and *Listeria* use the same receptors on U937 cells for attachment. A soluble binding assay was performed to determine if the same receptor on the surface of U937 cells was interacting with both bacteria. Figure 4 shows a Western blot of U937 cell proteins that were eluted using either GBS or *Listeria*. Three main proteins were found to be involved in attachment. Proteins with molecular masses of 60, 55 and 20 kDa were bound by both bacteria. These proteins appear to be involved in the attachment of both bacteria to U937 cells.

**DISCUSSION**

There is a large body of evidence showing that attachment and phagocytosis can take place in the absence of opsonins, suggesting that animals may rely on alternative mechanisms for recognition of evading pathogens by phagocytes (117). Nonopsonic recognition of bacteria is an important host defense mechanism especially in those individuals that are unable to produce sufficient amounts of complement and specific antibody, namely the immunocompromised and the neonates (148). Attachment of bacteria to macrophages is the first step in the phagocytic cascade; however, the mechanisms of attachment in an opsonin-independent environment are poorly understood. The overall goal of this project was to determine the mechanisms of opsonin-independent attachment that are involved in the recognition of GBS and *L. monocytogenes* by macrophages.

Previous work has shown that GBS (7,141) and *Listeria* (122)are able to adhere to macrophages in the absence of exogenous complement. In the present study *Listeria* was able to adhere to U937 cells (67) that were either untreated or PMA-treated. Treatment of U937 cells with PMA results in upregulation of certain receptors (103). The percentage of U937 cells with bacteria attached was similar in both cases suggesting that the receptor for *Listeria* is not upregulated with PMA treatment. Similarly, the receptor on U937 cells for GBS is not upregulated by PMA treatment (Chapter I).
Figure 4. Soluble binding assay. Western transfer of biotin-labeled U937 cell proteins that were bound by GBS and *Listeria*, detected using avidin-HRP and visualized with chemiluminescence. Lane 1 contains GBS-eluted U937 cell proteins, Lane 2 contains the GBS control, Lane 3 contains the *Listeria*-eluted U937 cell proteins, and Lane 4 contains the *Listeria* control. Molecular mass markers in kilodaltons are shown to the left.
Complement receptor type 3, CR3, is a member of the integrin family and has been suggested as a possible adhesin for both GBS (7,141) and Listeria (30). CR3 is the receptor for the complement component C3bi. Under opsonin-dependent conditions, C3bi coats the bacteria and increases the ability of macrophages to recognize the bacteria. However, CR3 has been shown to bind directly to type 1-fimbriated E. coli in the absence of complement, suggesting that CR3 may interact directly with bacterial ligands (117).

Antal et al. (7) and Sloan and Pistole (141) showed that GBS can bind to macrophages via the CR3 molecule. Because 100% inhibition was not observed with blocking monoclonal antibodies, there appeared to be additional receptors involved in GBS attachment. Using a bovine LAD system, I was able to show that GBS could bind to bovine PMNs with no CR3 surface expression almost as well as bovine PMNs expressing normal CR3 (Chapter II)

Listeria was able to bind to bovine PMNs regardless of CR3 expression. Only about 2% of BLAD PMNs express CR3 (Chapter II) and Listeria was able to adhere equally well to these PMNs and to normal PMNs suggesting the involvement of additional receptors. This is supported by work from Drevets and Campbell (30) who showed that listericidal macrophages use CR3 as the major phagocytic receptor to bind Listeria, but that CR3 mediates only a small amount of phagocytosis by a population of nonlistericidal macrophages, suggesting additional binding receptors for Listeria.

Listeria and GBS have similar adhesion patterns on U937 cells and bovine PMNs, which suggests that they may be binding to similar receptors. Competition studies supported this hypothesis. Preincubation of U937 cells with one organism inhibited the ability of the counterorganism to adhere. A similar model was reported by Lee et al. (90), who showed that C. albicans and P. aeruginosa bind to the same cell surface receptor on human respiratory epithelial cells. Using a similar competition assay, they showed that the pili from C. albicans were able to compete with the fimbriae from P. aeruginosa for binding
to buccal epithelial cells suggesting that they recognize the same receptors on the surface of these cells.

The competition experiments suggest that the same receptors from U937 cells are involved in the recognition of *Listeria* and GBS and this was confirmed with Western blot analysis. GBS and *Listeria* bound to the same molecular weight proteins from U937 cells. The proteins had molecular masses of 60, 55 and 20 kDa. The exact identity of the proteins is not known. Further analysis needs to be performed to determine their identities.

This study does show that GBS and *Listeria* recognize the same size proteins from U937 cells and that in competition studies one organism can inhibit the attachment of the other organism. These results could lead to beneficial treatment strategies for individuals infected with pathogenic organisms. If there are common receptors for nonpathogenic and pathogenic organisms it is possible that a treatment regime could include giving the infected individual an increased amount of nonpathogenic organisms to displace the pathogenic organism. In addition to the above mentioned line of defense, immunotherapeutics designed to treat one organism may be useful in the treatment of the other organism (90) if they bind to similar receptors on host cells.
CHAPTER IV

ADHERENCE OF GROUP B STREPTOCOCCI TO U937 CELLS INVOLVES A 21-kDa BACTERIAL PROTEIN

ABSTRACT

Group B Streptococcus binds to human macrophages in vitro in the absence of exogenous opsonins. The exact mechanisms that mediate this attachment are unclear. This study was undertaken to determine what protein adhesins are present on the surface of GBS that mediate attachment to macrophages. We have identified a 21-kDa protein from the envelope of GBS type III that binds directly to macrophages as determined by Western blot analysis. Antiserum against this protein inhibited attachment of GBS to macrophages by greater than 80% as measured by flow cytometry. Antiserum to the 21-kDa protein cross-reacted with 21-kDa proteins from GBS type Ib, type II, type III (COH31 and MR732) and type IV, as well as Staphylococcus epidermidis, but not GBS type Ia, Listeria monocytogenes or Enterococcus faecalis. These results suggest that the 21 kDa protein is important in mediating the attachment of GBS to macrophages in an opsonin-poor environment.

INTRODUCTION

Group B streptococci (GBS) are the leading cause of neonatal sepsis, meningitis and respiratory problems in the United States and are emerging as important pathogens in immunocompromised adults (36,132,157). Adhesion to host cells, initially epithelial cells for colonization and subsequently to phagocytic cells as part of the innate defense system, is a key event in the infectious process. The subcellular components on GBS and the host
cells involved in this recognition, however, have not been well characterized. Because the susceptible population may be compromised in their ability to produce protective levels of either complement or specific antibody, nonopsonic phagocytosis may be a major host defense mechanism in GBS infection. Previous studies in our laboratory have shown that murine macrophages (141) bind GBS in the absence of exogenous opsonins. In addition, GBS is able to bind to U937 cells in a dose-dependent manner as measured by ELISA and flow cytometry (Chapter I).

Many researchers have investigated the roles of bacterial proteins in the attachment process to host cells. Gaillard et al. (42) identified an 80-kDa surface protein on L. monocytogenes named internalin that enables these bacteria to bind and invade epithelial cells in vitro. Yersinia pseudotuberculosis invasin protein is a 103-kDa product found in the outer membrane that allows binding to mammalian cell receptors and enables the organism to enter the host cell (71). Group A Streptococci via their surface M protein adhere to particular host cell types (59). In all cases, there are specific molecules on both the bacteria and the host cells that are necessary for this attachment to occur (71).

Many studies have attempted to identify the GBS surface components that mediate adhesion to epithelial and phagocytic cells. In some studies lipoteichoic acid (LTA) has been shown to mediate adhesion of GBS to epithelial cells (109, 110), while others have shown that LTA has no effect on adhesion (104). Goldschmidt and Panos (51) found that GBS LTA was cytotoxic for growing cells and that a protein rather than LTA was primarily responsible for the adhesion of GBS type III to host monolayers in tissue culture. Other investigators (104, 151) have shown that GBS adherence to epithelial cells is sensitive to proteases, indicating that a surface protein may be necessary.

Lancefield and coworkers found that antibody to C-protein was capable of eliciting passive protection in mice with GBS strains expressing this protein (89). Madoff and colleagues have shown that antibody to the purified beta C-protein is both opsonic and
protective against strains of GBS that express the protein (97). Although, the C-protein is typically found on GBS strains belonging to serotype Ib, Ia and II GBS, it is not commonly found on type III GBS (16,73). Some investigators (93) have reported that anti-R protein antibodies protect mice against experimental infection with type II but not type III GBS strains. This study was undertaken to determine the role that GBS type III surface proteins play in the attachment of this organism to macrophages, initiating the first step in the phagocytosis cascade.

**MATERIALS AND METHODS**

**Bacterial Strains.** GBS strains originally characterized by the late Rebecca Lancefield were provided by Dennis Kasper, Channing Laboratory (Harvard Medical School). These strains are designated 090 (type Ia), H36B (type Ib), and 18RS21 (type II). COH31 (type III), M732 (type III) and a type IV strain, designated IV, were also donated by Dennis Kasper. Strains of *E. faecalis* (ATCC 19433), *S. epidermidis* (ATCC 12228) (American Tissue Culture Collection, ATCC; Rockville, MD) and a clinical isolate of *Listeria monocytogenes* (122) were also used. The GBS strains were maintained in our laboratory as a frozen stock in Todd Hewitt Broth (THB) (Difco Laboratories, Detroit, MI) with 10% dimethyl sulfoxide (DMSO). For use in these studies, the frozen stock was thawed and streaked on a blood agar plate. A single colony was then inoculated into 10-ml of THB and incubated overnight at 37°C. The additional bacterial strains were maintained as frozen stocks in T-soy broth with 10% DMSO. For use in these studies the strains were thawed and streaked onto T-soy (Difco) agar plates and single colonies were inoculated into T-soy broth and incubated overnight at 37°C.

**Cell lines.** U937 cells were obtained from ATCC. U937 cells were originally isolated from the pleural effusion of a 37-year old Caucasian male with diffuse histiocytic lymphoma (146). The U937 cell line is one of the only cell lines still expressing many of
the monocyte-like characteristics exhibited by cells of histiocytic origin. These cells were maintained at -70°C as frozen stocks in fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS) with 10% DMSO. In culture, these cells exist as non-adherent cells and were maintained in Dulbecco's minimal essential medium (DMEM) (Sigma, St. Louis, MO) supplemented with L-glutamine (Gibco, Gaithersburg, MD), penicillin/streptomycin (Gibco) and 10% FBS. Unless otherwise stated, prior to binding studies or soluble binding assays these cells were incubated overnight with 10^-8 M phorbol myristate acetate (PMA) (Sigma), which causes these cells to become adherent and to up-regulate certain cell receptors(129). In this activated state the cell are characteristically more macrophage-like (129).

**Biotin-labeling of U937 cells.** U937 cells were removed from the flask by agitation and washed three times with phosphate-buffered-saline (PBS). The cell suspensions were prepared fresh for each soluble binding assay, enzyme-linked immunosorbent assay (ELISA), or flow cytometry study. In some assays PMA-treated U937 cells were labeled with 100 μg/ml of biotin (Pierce, Rockford, IL) for 1 h at room temperature. The cells were washed extensively with PBS to remove unbound biotin. Freshly labeled cells were used to probe Western transfers of GBS lysates.

**Preparations of bacterial lysates.** A 10-ml overnight culture of bacteria was used to inoculate 200 ml of THB (GBS) or T-soy (other bacteria). The bacteria were grown at 37°C for 2 h to obtain exponential growth. The bacteria were harvested by centrifugation at 8000 x g for 10 min. In a glass beaker 30 ml of glass beads (0.45μm) was added to 50 ml of cell suspension in PBS. The suspension was mixed by swirling. Twenty milliliter aliquots were distributed into 50-ml conical tubes (Falcon, Fisher, Springfield, NJ) and each tube was agitated (Vortex Mixer, VWR, Bridgeport, NJ) at maximum speed for
approximately 10 min. The mixture was centrifuged at 300 x g to pellet the beads and the supernatant fluid containing the bacterial lysates was collected. The samples were dispensed into 1-ml aliquots and stored at -20°C.

A time course experiment was also performed to determine the optimal growth period for GBS for maximum protein expression. THB (10 ml) tubes were inoculated with 1 ml overnight GBS culture and the tubes were incubated for various times. The cells were harvested and lysates prepared as described above.

**Biotin-labelling of GBS (COH31) surface proteins.** Two hundred milliliters of GBS were grown as described above. The bacteria were harvested by centrifugation at 8000 x g for 10 min. The bacteria were washed 3 times with PBS followed by centrifugation at 8000 x g for 10 min. After the final wash the bacteria were resuspended in 10 ml of PBS. To the bacterial suspension, 0.1 mg/ml of biotin, dissolved in DMSO, was added and the mixture incubated in the dark for 1 h with gentle rocking. The bacteria were washed 3 times to remove unbound biotin. Bacterial lysates were prepared as described above.

**Identification of surface-expressed envelope proteins of GBS (COH31).** Biotin-labeled envelope proteins of GBS were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)(87). The proteins were separated on 12% acrylamide gels using a Hoefer™ gel system (Hoefer Scientific Instruments, San Francisco, CA). The separated proteins were transferred to nitrocellulose as described by Towbin (150) using a Biorad™ transfer unit (Hercules, CA). The nitrocellulose was blocked with 5% skim milk (Difco) for 1 h. After blocking, the nitrocellulose was incubated with avidin-horseradish peroxidase (HRP) (Biorad) (1:1000 dilution) in 1% skim milk buffer for 1 h with gentle rocking. The nitrocellulose was washed 3 times for 5 min
each in PBS. The biotin-labeled surface proteins were visualized using 4-chloro-naphthol (Biorad). Unlabeled GBS envelope proteins were used as a negative control.

**Identification of GBS (COH31) proteins that bind to U937 cells.** Unlabeled GBS lysates were separated on 12% acrylamide gels. The proteins were transferred to nitrocellulose and blocked as described above. Approximately $1 \times 10^8$ biotin-labeled, PMA-treated U937 cells in PBS were added to the nitrocellulose and the mixture was incubated for 2 h at 37°C with gentle rocking. The nitrocellulose was washed 3 times with PBS to remove any unbound U937 cells, then incubated with avidin-HRP (1:1000 dilution) for 1 h at 37°C. The nitrocellulose was washed 3 times with PBS to remove unbound avidin-HRP and the GBS proteins that were bound directly by U937 cells were visualized with 4-chloro-naphthol.

**Soluble binding assay.** One confluent T-75 flask of U937 cells (unstimulated) was washed 3 times with PBS. After the last wash, the pellet was resuspended in 500 μl of 5% gelatin in PBS and incubated for 2 h at 37°C. At the same time, 200 μl of a GBS (COH31) membrane preparation, previously labeled with biotin, was incubated in 800 μl of 1% Triton X-100 in PBS for 2 h at 37°C. For each assay the U937 cells were pelleted in two separate tubes each containing the same volume of cells. One pellet was resuspended in the GBS mixture; the other pellet was resuspended in 1 ml of Triton X-100 in PBS and served as the negative control. The tubes were incubated for 24 h at 37°C with gentle rocking. Each tube was washed 4 times with 1 ml of 1% Triton X-100 in PBS. The GBS proteins bound to the U937 cells were eluted with Laemmli sample buffer (50 μl) and the solution was boiled for 1 min. The negative control tube was similarly treated. The tubes were centrifuged (Microfuge™ II, Beckman, Fullerton, CA) at maximum speed and the supernatant fluids were collected. The samples were separated on 12% SDS-PAGE.
minigels. The separated proteins were transferred to nitrocellulose and blocked with 5% skim milk buffer. Avidin-HRP (1:1000 dilution) was added and the mixture incubated for 1 h at room temperature. The nitrocellulose was washed 3 times for 5 min each in PBS. GBS proteins that bound directly to U937 cells in solution were visualized with chemiluminescence (Amersham, Arlington Heights, IL).

**Preparation of Antisera.** The 21-kD GBS (COH31) envelope protein was used as the immunogen. The protein was purified using preparative SDS-PAGE. The proteins were excised from the gel and eluted from the gel matrix using a Centrilitor™ (Amicon®, Beverly, MA). Each gel fragment provided enough protein for the immunization of one rabbit. The protein was emulsified in TiterMax® (CytrRx® Corp, Norcross, GA) and injected subcutaneously into New Zealand White rabbits (Millbrook Farms, Amherst, MA). The rabbits were boosted at two-week intervals, three times, and the animals bled to determine titers. Rabbits with a titer above 1000 were cardiac bled. The blood was collected in serum tubes (Beckon Dickinson, San Jose, CA), and the serum was separated by centrifugation, aliquoted into 15-ml tubes and stored at -20°C.

**Antisera titer determination.** Titers of the antibody to the 21-kDa protein were determined by ELISA. A 10-ml culture of GBS (COH31) was grown for 2 h as previously described. The cells were washed 3 times in PBS and reconstituted in 10 ml of PBS after the final wash. Approximately 1 x 10^8 bacteria were added to each well of a 96-well ELISA plate and dried overnight. The plates were blocked with 5% BSA/PBS for 1 h. Pre-immune serum and immune serum were titrated in triplicate. Bound antibody was detected with goat anti-rabbit antibody conjugated to HRP (Pierce) (1:5000). The antibody titer was defined as the reciprocal of the serum dilution that resulted in an A_{405} of >.200.
Identification of 21 kDa protein from additional bacterial strains. The ability of the anti-21 kDa antiserum from GBS to cross-react with additional bacterial strains was determined by Western blot analysis. Membrane preparations from each bacterial strain were separated on SDS-PAGE and transferred to nitrocellulose. The filter was blocked with 5% milk buffer and the blots were incubated in the appropriate antiserum (1:250 dilution). Pre-immune serum was used as the negative control. The blots were washed and incubated with goat anti-rabbit antibody-HRP (1:1000). After the secondary antibody was removed with washing the proteins were visualized with chemiluminescence.

Inhibition Assay. The rabbit anti-GBS antibody was tested for its ability to inhibit attachment of GBS to U937 cells. GBS were labeled with FITC (Chapter 1) and ~ 1 x 10^8 bacteria were incubated with various concentrations of antiserum. Labeled GBS alone or GBS incubated with preimmune serum served as a maximum binding control. The mixture of GBS and antiserum (or GBS alone) was incubated for 2 h at 37°C. Fifty microliters of GBS-antiserum mixture was incubated with 1 x 10^6 U937 cells for 30 min. The unbound bacteria were removed with 2 washes of PBS and the cells were fixed with 0.5% paraformaldehyde. The samples were read on a Facscan™ flow cytometer and data analysis was performed using the Lysis II™ software. The % inhibition was calculated using the following formula:

\[ \text{% inhibition} = \frac{100 - (\text{median binding of the test/median binding of the control \times 100})}{\text{test/median binding of the control}} \]

RESULTS

Optimal protein expression from GBS. Maximum GBS protein expression from different culture incubation times was examined. Figure 1 shows GBS proteins separated on SDS-PAGE and stained with Coomassie blue. The lysates were prepared from GBS cultures incubated at different times and protein expression was examined. This assay
Figure 1. Protein expression at various time points in the growth of GBS. Envelope preparations of GBS were made at various growth points. Lane 1 contains preparations made at 4 h, lane 2-3.5 h, lane 3-3 h, lane 4-2.5 h, lane 5-2 h, lane 6-1.5 h, lane 7-1 h, lane 8-0.5 h, and lane 9 contains the molecular mass markers in kilodaltons. The SDS-PAGE gel was stained with Coomassie blue.
shows that a 2-h incubation time provides maximal protein expression and all subsequent
GBS cultures were incubated for 2 h. Figure 2 shows various concentrations of GBS
proteins separated on SDS-PAGE and stained with Coomassie blue. This experiment was
performed to determine the optimal concentration of GBS proteins to use in the following
experiments. The optimal concentration of GBS proteins used in the following
experiments was between 50 and 25 μg.

**GBS attaches to U937 cells via a 21-kDa surface protein.** GBS were biotin-
labeled and lysates of these bacteria were resolved by electrophoresis. The proteins were
transferred to nitrocellulose, then probed with streptavidin to visualize the biotin-labeled,
and thus, surface protein (Fig 3). As expected, a number of proteins on the surface of
GBS were detected.

We employed two separate assays to determine if a particular GBS surface protein
could specifically bind to U937 cells. In the first assay unlabeled GBS proteins separated
on SDS-PAGE and transferred to nitrocellulose, as above, were incubated with U937 cells
that had been labelled with biotin. Fig. 4 shows that a 21-kDa protein from GBS binds to
U937 cells. The protein detected by this method appears to be a surface protein when
compared to the proteins appearing in Fig 3 (see arrow). To be sure that the protein
detected was in fact a surface protein a soluble binding assay a soluble binding assay was
performed.

In the soluble binding assay the GBS was biotin-labeled before the bacteria were
disrupted. These labeled-bacterial lysates were incubated with whole, unlabeled U937
cells and the bacterial proteins allowed to attach to the whole cells. The GBS proteins were
dissociated from the U937 cells, separated on a SDS-PAGE gel, and transferred to
nitrocellulose. Fig. 5 shows a Western transfer of GBS proteins that bind to U937 cells.
In this assay we detected 3 proteins: 100-kDa, 25-kDa, 21-kDa. Since the bacterial

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Figure 2. GBS envelope preparations. Various concentrations of GBS envelope preparations were separated on SDS-PAGE and stained with Coomassie blue. Lane 1 contains the molecular mass markers in kilodaltons. Lane 2 contains 100 µg, lane 2 contains 50 µg, lane 3 contains 25 µg, lane 4 contains 10 µg, lane 5 contains 5 µg, lane 6 contains 1 µg and lane 7 contains 0.5 µg of GBS envelope preparations.
Figure 3. Western transfer of separated GBS envelope proteins. Biotin-labeled GBS envelope proteins were separated on SDS-PAGE and transferred to nitrocellulose to detect surface associated GBS proteins. Lane 1 contains the pre-stained molecular mass standards in kilodaltons. Lanes 2-7 contain different envelope preparations of GBS (COH31) at 25 μg/ml. The protein that was directly bound by U937 cells is indicated with the arrow.
Figure 4. Western transfer of separated GBS envelope proteins probed with biotin-labeled U937 cells. Unlabeled GBS envelope proteins were separated on SDS-PAGE and probed with labeled U937 cells to determine the GBS proteins that interact directly with U937 cells. Lanes 1 and 8 contain pre-stained molecular mass markers in kilodaltons. Lanes 2-7 contain different envelope preparations of GBS (COH31) at 25 μg/ml.
Figure 5. Soluble binding assay. Envelope preparations of biotin-labeled GBS were incubated with U937 cells. The GBS proteins that bind directly with U937 cells were resolved by electrophoresis, transferred to nitrocellulose, and probed with streptavidin. Lane 1 contains labeled GBS envelope proteins that bind U937 cells directly. Lane 2 contains the negative control. Molecular mass markers in kilodaltons are indicated on the left.
proteins had been biotin-labeled prior to disruption (21), I concluded that the proteins detected were surface-associated. The 21-kDa protein appeared in both of the detection assays; therefore, I focused on it.

**Reactivity of rabbit antiserum to the 21-kDa protein.** The antiserum against the 21-kDa surface GBS protein had a titer of 4250 against whole bacteria as measured by the ELISA assay (data not shown). Fig. 6 represents a Western transfer of separated bacterial lysates from a number of bacteria that were probed with the antiserum to the 21-kDa protein. The antisera cross-reacted with a 21-kDa protein from GBS type Ib, type II, type III (COH-31 and MR732) and type IV, as well as *S. epidermidis*, but not GBS type Ia, *L. monocytogenes* or *E. faecalis*.

**Anti-GBS 21-kD antiserum inhibits binding of GBS to U937 cells.** To determine if the 21-kDa protein is important in the binding process, we tested whether the corresponding antiserum could inhibit GBS attachment to U937 cells. In flow cytometry studies the anti-21-kDa antiserum significantly inhibited GBS (COH31) attachment to U937 cells as compared to pre-immune antiserum (Fig. 7). At the highest concentration of antisera used I was able to achieve an 80% reduction in the attachment of GBS to U937 cells. The data are represented as percent inhibition.

**DISCUSSION**

Studies with group A streptococci and certain host cells have shown that the adhesion is mediated by the streptococcal M protein (26,154). With *Streptococcus mutans* adhesion is mediated by the adhesin antigen I/II (108). This study was undertaken to determine what role surface proteins of type III GBS have on adhesion to macrophages.

Previous observations have indicated that GBS may attach to host cells via surface
Figure 6. Western transfer of bacterial envelope proteins probed with rabbit anti-21 kDa GBS protein antiserum. The bacterial proteins were separated using SDS-PAGE and transferred to nitrocellulose. The antiserum was used to detect cross-reactive proteins from these bacterial preparations. Lane 1 contains prestained molecular mass standards in kilodaltons. The remaining lanes contain separated envelope preparations from the following bacterial strains: lane 2 GBS type III (COH31), lane 3 GBS type Ia (090), lane 4 GBS type II (18RS21), lane 5 E. faecalis, lane 6 GBS type IV, lane 7 GBS type Ib (H36), lane 8 S. epidermidis, lane 9 GBS type III (MR732), and lane 10 L. monocytogenes.
Figure 7. Inhibition Assay. Rabbit anti-21 kDa GBS protein antiserum was used at various dilutions to determine if it could inhibit the binding of GBS to U937 cells. This was measured using flow cytometry. Each sample was an average of two replicates and the data are represented as the percent inhibition of GBS binding to U937 cells. Pre-immune serum was used as the negative control.
proteins. Tamura et al. (147) showed that GBS surface proteins appear to play a critical role in adherence to epithelial cells in vitro, while LTA and capsular polysaccharides did not appear to be involved. In addition, other studies have suggested that antibodies against GBS surface proteins may also play an important role in protection against GBS disease (39, 101, 152).

In this study a 21-kDa protein from the surface of GBS that directly binds to U937 cells was identified. Antisera against this protein inhibited attachment of GBS to U937 cells by up to 80%, as measured with flow cytometry. Since I was unable to achieve 100% inhibition with the antiserum, this indicated that there may be additional interactions between GBS and U937 cells. This is supported by the work of Nealson and Mattingly (109), who showed that purified LTA was able to significantly inhibit adherence of GBS to embryonic cells. Because 100% inhibition could not achieved, they suggested that attachment is likely a multi-step process and that other receptors are involved. Hasty et al. (60) have proposed a multi-step adhesion process for Streptococcus to host cells. In their model the first step of adhesion is relatively weak and reversible, probably mediated by hydrophobic interactions such as LTA. However, the second step may involve adhesins that are specialized receptors expressed by cells within certain niches (such as M protein on S. pyogenes). This second interaction is considered to be a stronger one. We believe that we have identified a protein that falls into the second category. The first step in the proposed model by Hasty et al. is easily dissociated with multiple washing steps. In our assay systems, we perform multiple washes after the bacteria and macrophage have interacted that would easily dissociate a weak interaction.

The rabbit anti-21-kDa antiserum was able to recognize 21-kDa proteins from the surface of multiple GBS strains. This antiserum, however, did not interact with proteins from the surface of Listeria monocytogenes or Streptococcus faecalis, which suggests that these bacteria have other adhesins for macrophages. Recent studies with L.
*monocytogenes* have described a listerial surface protein ActA that is a major virulence factor and is a bacterial ligand in the recognition of receptors on macrophages (62,96). This antiserum did not react with type Ia GBS. The reason for this is not known.

In addition to type-specific antigens from GBS, two additional surface proteins, C and R, have been described as potential virulence factors for GBS. The C-proteins have been found on all serotype I b and many Ia and II GBS, but they are uncommon on type III GBS (16,73). The molecular mass of the C-protein has been reported to range from 14-kDa to several hundred. However, the banding pattern for C-protein as seen on SDS-PAGE is commonly a ladder pattern with proteins at 8-kDa intervals (101). It is therefore unlikely that our protein is a C-protein. Similarly, it is unlikely that our protein is a member of the R-protein family. The R-protein has been shown to be heterogeneous on SDS-PAGE, with the main protein band at a molecular mass of greater than 100-kDa(37). This protein has also been shown to display a ladder pattern on SDS-PAGE, which was not observed with our protein.

In conclusion, I believe that the 21-kDa surface protein of GBS is an important mediator of attachment to macrophages. Further studies are needed to identify the counterreceptor from macrophages involved in this adhesion. Recent studies by Valentin-Weigand *et al.* have shown that GBS are able to enter and survive inside J774 macrophages (151); however, the molecular mechanisms have not been determined. I would like to determine if blocking the 21-kDa protein results in decreased phagocytosis by the macrophage. It is possible that the 21-kDa protein is also associated with this process.
OVERALL DISCUSSION

Attachment of microorganisms to host cells and subsequent phagocytosis are important host defense mechanisms. The receptors engaged on the macrophage by the microorganism determine its fate. Internalization is initiated by the interaction of specific receptors on the surface of the phagocytes with ligands on the surface of the microorganism (4). Although adherence is a separate process from host cell entry, if the receptor to which the pathogen binds is an endocytic or phagocytic receptor the pathogen will be internalized, without additional requirement (4). Aside from their ability to recognize a eukaryotic address, adhesins play a substantial role in determining the outcome of a prokaryotic-eukaryotic interaction (63). Engagement of certain macrophage receptors may lead to internalization and subsequent survival of this same pathogen, while engagement of a different receptor can lead to internalization and killing of the pathogen (30). Regardless of the outcome, the first interaction between the pathogen and the macrophages is attachment. Bacterial attachment to macrophages was the focus of this study. The goal of the present study was to identify the adhesins on macrophages that recognize group B streptococci (GBS) in opsonin-independent environments and to identify the ligand(s) on GBS recognized by macrophages.

Opsonin-independent recognition of bacteria by macrophages is not completely understood. The susceptible population for infection with GBS is the immunocompromised and the neonate. In these populations complement and specific antibody may be decreased or absent. Therefore, there must be recognition of the pathogen by the macrophage under opsonin-independent conditions and this is the premise for the
present study.

The test model chosen to investigate prokaryote-eukaryote adhesion was the U937 cell model (146). This cell line has been used extensively to explore the functions of macrophages (18,67,69,102) and served as an appropriate model to study the interaction of GBS with macrophages in a complement-independent environment. GBS were able to adhere to U937 cells in a dose-dependent manner. Stimulation of U937 cells with phorbol esters, which leads to upregulation of membrane receptors (24,65,70), did not increase binding of this organism to U937 cells, indicating that the receptor for these bacteria is not upregulated.

Complement receptor type 3, CR3, the receptor that under opsonin-dependent conditions binds to C3bi-coated bacteria (4), has also been shown to bind bacteria in opsonin-independent environments. Studies with *E. coli* (117) have shown that this organism binds directly to CR3 under complement devoid conditions. *Listeria* is also able to adhere to CR3 under the same conditions (S. Maganti, unpublished). Using blocking antibodies to the complement receptors, we showed that CR3 was involved in the binding of GBS to U937 cells, but 100% inhibition was not achieved suggesting the role of additional receptors. This is supported by Antal *et al.* (7) and Sloan and Pistole (141), who showed that CR3 is partially involved in GBS attachment to macrophages under opsonin-independent conditions.

To further explore the involvement of CR3 we used a bovine leukocyte adhesion deficiency model. Leukocyte adhesion deficiency is a disease that is characterized by a decrease in the surface expression of $\beta_2$ integrins on leukocytes (143,144). The genetic and clinical manifestations of this disease have been extensively studied in cows (48,76,78,79). This model has been used to study *Leishmania-major* macrophage interactions (126), providing precedence for use of this model to investigate CR3 involvement in bacterial attachment. Both GBS and *Listeria* were able to bind to normal,
carrier and BLAD PMNs in a dose-dependent manner. There was better binding to the normal PMNs compared to the BLAD suggesting that CR3 may be required for optimal binding but that this receptor is not a requisite for GBS and Listeria attachment. These results are supported by my previous findings that CR3 blocking antibodies only partially inhibit binding of GBS to U937 cells and the studies by Antal et al. (7) and Sloan and Pistole (141). Drevets and Campbell showed that listericidal macrophages use CR3 as the major phagocytic receptor to bind Listeria, but that CR3 mediates only a small amount of phagocytosis by nonlistericidal macrophages, suggesting additional binding receptors for this bacteria (30).

The binding of GBS and Listeria to both U937 cells and bovine PMNs was similar suggesting that these organisms may be interacting with the same or similar receptors on phagocytes. Results from an ELISA competition assay showed that Listeria is able to block attachment of GBS to U937 cells and that GBS can block attachment of Listeria to U937 cells. This study suggests that GBS and Listeria are interacting with the same receptor on U937 cells. This was consistent with the work of Lee et al. (90). Using a similar competition assay, they showed that the pili of Pseudomonas aeruginosa and the fimbral adhesin of Candida albicans express a structurally conserved binding domain that enables them to bind to the same cell surface receptor on human respiratory epithelial cells.

The results from the present study indicate that Listeria and GBS interact with U937 cells via the same receptor. Indeed, 3 proteins from the surface of U937 cells were shown to interact with GBS and Listeria as detected in the soluble binding assay. The proteins have molecular masses of approximately 60, 55 and 20 kilodaltons. In the same assay, GBS was shown to interact with a 55-kDa protein from PMNs of normal, carrier and BLAD cows. Thus, a 55-kDa protein binding to GBS was identified in both U937 cells and bovine PMNs. This suggests that GBS and Listeria bind to the same receptors on the surface of U937 cells and that these receptors may be evolutionary conserved. Further
work needs to be done to determine the identity of these proteins.

There are a number of proteins on the surface of GBS that have been implicated as having immunological relevance in GBS disease (37,93,96); however, to date the protein from GBS that interacts with macrophages has not been determined. The focus of this study was to identify the protein(s) from GBS that interact(s) with macrophages in an opsonin-independent environment. A 21-kDa protein from GBS was found to interact directly with U937 cells as determined by Western blot analysis. Antisera against this protein significantly inhibited attachment of GBS to U937 cells suggesting that this protein may be the adhesin for macrophage attachment. In addition, the antisera was found to cross-react with a number of GBS strains as well as additional Gram-positive bacteria, suggesting that this protein may be conserved in some Gram-positive bacteria. This study indicates that the 21-kDa protein from GBS is the adhesin for macrophages.

In conclusion, it appears that GBS and *Listeria* bind to the same proteins on U937 cells. Based on molecular mass, these proteins are not of the $\beta_2$ integrin family. The results suggest that while the $\beta_2$ integrins may be involved for optimal adhesion of these bacteria, they are not the primary receptor responsible for recognition. The interaction of $\beta_2$ integrins may be at the level of internalization (7,30) of these organisms and not adherence. In addition, a 21-kDa protein from GBS was identified and appears to be the adhesin for macrophages. This protein may be conserved in some Gram-positive organisms and is involved in recognition.
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