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Sloan, Anne Ragonese, Ph.D.

University of New Hampshire, 1992

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THE ROLE OF LIPOTEICHOIC ACID AND THE LEOKOCYTE INTEGRINS IN THE BINDING OF GROUP B STREPTOCOCCI TO MURINE PERITONEAL MACROPHAGES

BY

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DISSERTATION

Submitted to the University of New Hampshire
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the Requirements for the Degree of

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in

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This dissertation has been examined and approved.

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THE ROLE OF LIPOTEICHOIC ACID AND THE LEUKOCYTE INTEGRINS IN THE BINDING OF GROUP B STREPTOCOCCI TO MURINE PERITONEAL MACROPHAGES

by

Anne R. Sloan

University of New Hampshire, May, 1992

The macrophage has been shown to bind potentially pathogenic bacteria in the absence of serum components, but the mechanism is not well understood. The macrophage is a key component of our non-inducible defenses and this form of innate immunity is of particular importance for individuals in which the immune system may be sub-optimally functional, *e.g.*, neonatal and geriatric individuals, and for sites in the body in which serum enhancement through opsonins is thought to be insufficient, *e.g.*, lungs. To understand the mechanism by which mammalian innate host defenses respond to potential microbial aggression by opportunistic microorganisms, I developed two assays to quantify the *in vitro*, serum-free adherence of group B *Streptococcus* to murine peritoneal macrophages. The first is a modified direct microscopic assay, while the second is based upon an

enzyme-linked assay using bacterial specific antibody.

Sugar inhibition assays as well as studies with isogenic strains and neuraminidase-treated streptococci could not confirm a mechanism proposed by other investigators that macrophages recognize these streptococci by a lectin-like receptor for galactose. I observed that binding characteristics of the macrophage, such as temperature- and divalent cation- dependence, protease sensitivity, and enhancement by fibronectin and phorbol esters, were similar to those of the β_2 integrins, a heterodimeric family of leukocyte receptors. Monoclonal antibodies M1/70, which recognizes the α-subunit of one member of the family, complement receptor 3 (CR3), and M18/2, which recognizes the β-subunit, common to all three members, were each shown to reduce bacterial adherence in down-modulation, flow cytometric and soluble inhibition studies. Using similar approaches I have additionally shown that the purified streptococcal membrane phosphosugar, lipoteichoic acid, can inhibit the binding of these bacteria to macrophages. These results support roles for both lipoteichoic acid and the β_2 integrins in the innate recognition of group B streptococci by macrophages.

INTRODUCTION

Phagocytic cells in higher animals are capable of discriminating between foreign material and self components, as well as between self and altered self (47). This recognition ability is thought to have developed with the evolution of multicellular life forms, since primitive colonial marine species such as *Porifera* exhibit the ability to exclude non-self life forms (105). Concomitantly the generalized process of phagocytosis became associated with specialized cells in metazoans (26). Hemocytes from invertebrates have been shown to perform many of the functions associated with phagocytic cells in well-studied mammalian systems (67).

In vertebrates a highly adaptive immune system has evolved, involving extensive cooperation between cellular and humoral components and the concept of specialized recognition (84), and this facet of the host's defenses has been the subject of comprehensive studies. The more primitive forms of phagocyte recognition appear, however, to have been retained and the cells appear capable of recognizing foreign particulate material without the use of their more recently evolved receptors for the Fc component of the immunoglobulin molecule or the C3b component of the complement system (94).

The mechanisms that underlie these highly important recognition

abilities of phagocytes is not fully understood. It is not known if the binding of a bacterium to a phagocytic cell involves complex stereospecific receptors in the cell for components of the cell surface of the microorganism or whether the binding depends on a simpler, relatively nonspecific ability to recognize carbohydrate or other cell surface constituents. Such interactions may depend on hydrophobic molecules present in the foreign particle inserting themselves in the phospholipid cell membrane of the phagocytes (109).

Cell membranes contain lipids, proteins, oligosaccharides and polysaccharides in various combinations (91). Membrane phospholipids have hydrophilic polar heads that interact with water at the external and internal sides of the phospholipid bilayer. Their hydrophobic tails associate within the bilayer and are excluded from water. These molecules are capable of lateral movement in the membranes. Membrane proteins exist in hydrophilic and hydrophobic forms. The hydrophobic proteins (integral membrane proteins) are embedded to variable depths into the phospholipid bilayer and may span the membrane. In the latter situation they will have hydrophilic peptide sequences at either end of the molecule. Such molecules often have a carbohydrate chain at the external surface of the bilayer and are glycoproteins. Other non-structural proteins with hydrophilic properties are present at the external and internal surfaces of the lipid bilayer and are called peripheral membrane proteins.

Peripheral membrane proteins can be dissociated from integral membrane

proteins and glycolipids by high salt concentration and chelating agents (33). Their removal, in contrast to the integral proteins, usually does not affect the structural integrity of the cell membrane. Some integral proteins can, like the phospholipids themselves, move laterally in the membrane. Membrane glycoproteins and glycolipids are arranged so that their carbohydrate residues are exposed at the external surface of the membrane. Many of these molecules are known to act as receptors for antibodies, hormones, viruses, and other agents. Their lateral movement can result in the formation of patches or caps in which their distribution is altered from a diffuse to a coalesced arrangement.

Microtubules together with microfilaments lie in the cytoplasm of the cell and control the mobility and distribution of cell surface receptors (62). The fact that different integral proteins and glycoproteins have considerably different rates of lateral movement suggests the importance of restricted mobility of some components so as to maintain an ordered display of patterns on the cell surface. Such cell-specific surface patterns could constitute an important mechanism for determining cell contact and recognition phenomena. The ability of some of these molecules to move rapidly in the cell membrane in response to environmental factors, such as complement or antibodies, enables reversible changes in the cell surface pattern to take place.

Non-opsonin-mediated recognition of bacteria by macrophages

Serum factors have been shown to enhance the rate of uptake of microorganisms by phagocytes since the early 1900's (111). Over the years it has been established that the primary role of the opsonins is to provide a means of recognition between phagocytes and their targets, *e.g.*, bacteria (33, 50) in a process known as opsonophagocytosis.

Opsonophagocytosis can occur in three modes (82). In the first, only antibodies participate by reacting via their $(Fab)_2$ combining sites with the appropriate antigenic determinants on the bacterial surface. The Fc portion of the coating immunoglobulin G molecule binds to the corresponding Fc receptors on the surface of the phagocytes, thereby bringing the microbe and phagocytic cell in close proximity. In the second mode, only the C3b and C3bi fragments of complement participate. The C3b and C3bi fragments are generated from C3 as a result of its activation by contact with certain microbial surface constituents, a process known as the alternative pathway of activation. C3b or C3bi attaches to the microbial surface covalently via thiol ester or amide bonds and to the phagocyte surface via specific receptors known as CR1 or CR3, respectively (50).

In the third mode, both antibody and C3b (or C3bi) participate. The antibody binds to the microbial surface antigens, and the antigen-antibody complexes thus formed activate complement, either by the classical pathway or the alternative pathway, following which C3b or C3bi is deposited onto the

microbial surface. The microorganisms, which are now coated with both antibody and complement fragments, bind to the phagocytes via the receptors for both ligands. This binding is greatly enhanced over that with either immunoglobulin G or the fragments of C3 alone (52).

The second mode of opsonophagocytosis has been a subject of much interest, since it can occur in the non-immune host, and therefore serves as an early defense mechanism against microbial infections (25). It depends on the availability of complement at the site of infection, on the ability of bacteria to activate complement by the alternative pathway and to deposit fragments of C3 on their surfaces, and on the proper orientation of the cell-bound fragments to react with the appropriate receptors on the phagocytes. These conditions are not always met. Certain sites, such as the lungs (28, 51) or renal medullae (85, 86) have low serum opsonin concentrations, which may not be sufficient to effectively opsonize many bacterial species. Additionally, not all organisms activate the alternative pathway efficiently (25, 42, 66), and complement-deficient states have been reported during the neonatal period (1, 15). Moreover, patients deficient in C3 can frequently cope with a variety of bacterial infections, irrespective of the ability of the bacteria to activate the alternative pathway.

Evidence from *in vitro* studies demonstrates that phagocytosis of bacteria occurs in opsonin-free media (20, 33, 43, 53, 69, 70, 101), while that from *in vivo* studies shows clearance of bacteria from the lungs by alveolar

macrophages (28, 51) or from the blood by the reticuloendothelial system of animals depleted of complement (11). Nonopsonic recognition between bacteria and phagocytes may be occurring by one or more mechansims.

Several investigators have postulated that the net surface charge or the hydrophobicity of particles or bacteria determines whether they can be bound by phagocytes (92, 102), but no direct evidence for these assumptions has been obtained. It is still possible that nonspecific or ill-defined surface properties may account for the recognition by phagocytes of some particles (*e.g.*, polystyrene beads). However, as pointed out by Griffin (33), recognition of most physiologically relevant particles must involve more specific mechanisms.

The role of lectins in recognition of microorganisms by phagocytic cells

During the last decade considerable evidence has accumulated showing that specific recognition by phagocytes may be accomplished by the interaction of carbohydrate-binding proteins, *e.g.*, lectins, on the surface of one type of cell that combine with complementary sugars on the surface of another in a lock-and-key manner (82, 83). This type of recognition, which also leads to phagocytosis, has been termed lectinophagocytosis (60). Lectinophagocytosis of bacteria can occur in two major modes. In the first, bacteria that carry surface lectins bind to complementary carbohydrates on the surface of the phagocytic cells. In the second, lectins that are integral components of the

phagocytic cell membrane bind to carbohydrates on the bacterial surface.

Macrophages have several cell-associated lectins that have been implicated in microbial recognition (30). A receptor on human macrophages has been shown to recognize mannose-bearing pathogens and to mediate their internalization (24). This mannose/fucosyl receptor (MFR) recognizes *Leishmania* promastigotes and synthesis of reactive oxygen metabolites is triggered by adherence to this receptor (46). Human monocytes bear β-glucan receptors that react with zymosan particles (19), thus providing the cells with the ability to detect microorganisms bearing this glycan in the absence of serum factors. The clearance of *Klebsiella pneumoniae* from the lung may be due to a mannosyl/N-acetyl-D-glucosaminyl receptor found on alveolar macrophages (7).

Rat Kupffer cells *in vitro* strongly bind neuraminidase-treated but not untreated rat erythrocytes (49). Preincubation of the macrophages with D-galactose and related sugars inhibits cell adherence and suggests that the galactose-specific lectin receptor recognizes senescent erythrocytes (79).

The Group B Streptococcus

Group B streptococci (GBS) are a major cause of neonatal meningitis and septicemia in this country (80). These organisms are classified into serotypes based on their specific capsular polysaccharides. The capsular polysaccharides of serotypes Ia, Ib, II and III have been purified and characterized (39, 38, 41,

40). The structure of the capsular polysaccharides for these serotypes is represented in Fig. 1 (44). The backbone antigen is composed of a repeating unit of two to five monosaccharides, depending on the serotype. Side-chain βD-galactose may be either exposed or be covered by a terminal sialic acid.

Type II strains, which account for ~15% of all infant infections, have been isolated only rarely from infants with meningitis (108, 110). Among GBS isolates from adults with meningitis, however, type II strains predominate (110). Of the defined capsular polysaccharides the type III capsule is found on organisms most frequently isolated from neonatal infections. Although frequently found among the flora colonizing the vaginal tract, they seldom contribute to disease in the otherwise healthy adult host. During parturition, particularly when accompanied by premature rupture of the fetal membranes, GBS may be acquired by the newborn; systemic infection may follow (35).

Role of complement receptor 3 (CR3) in host defenses

Adhesion between phagocytes and other cells is a necessary prerequisite for phagocytosis (103). Phagocytosis represents an important effector mechanism for the eradication of infectious agents and is performed primarily by specialized cells of two different lineages, namely polymorphonuclear neutrophilic granulocytes (PMN) and mononuclear phagocytes (Mø). To fulfill their role in host defense, PMN and Mø must respond to specific signals that indicate the presence of infectious agents and

Structural modifications of group B streptococcal polysaccharides

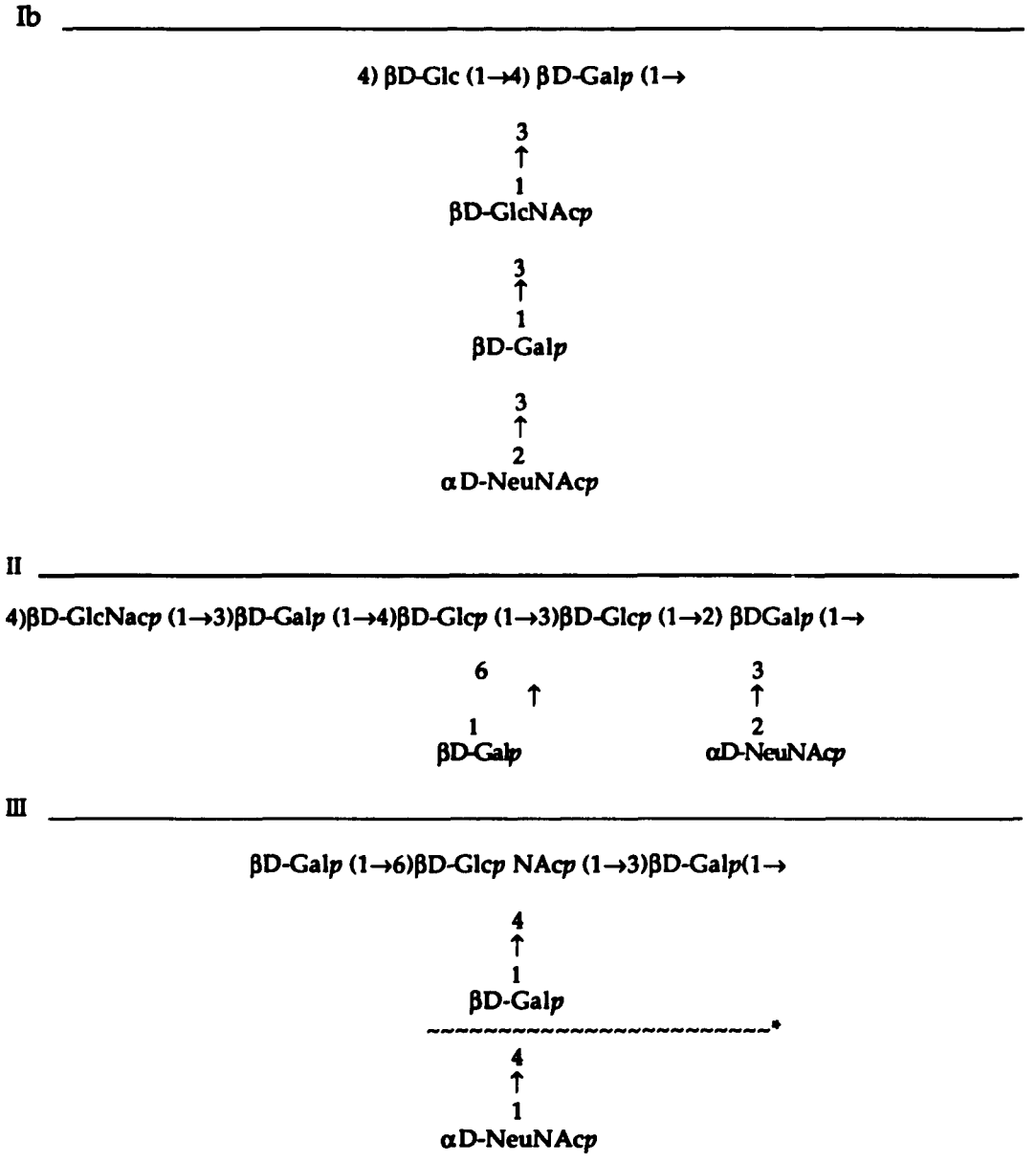

Fig. 1. Structure of GBS serotypes with modification by neuraminidase treatment (2), from reference 44.

* site of cleavage by neuraminidase

also collaborate with the elements of specific immunity. It has long been assumed that these cells have specific receptors for binding microorganisms directly, but only recently have such receptors been isolated and characterized.

The idea that a few receptors could be responsible for a wide range of adhesion events first arose with the discovery of a class of patients that exhibit recurrent life-threatening infections with gram-positive, gram-negative and fungal pathogens (5). These patients exhibit extreme leucocytosis but fail to form pus at sites of infection. *In vitro* experiments showed that PMN from these patients are defective in adhesion to C3bi-coated erythrocytes, to protein-coated glass or plastic surfaces (3), and to endothelial cells (34), and that failure to adhere results in the failure to display chemotactic responses. Thus, susceptibility to infection probably results both from an inability to bind and ingest opsonized pathogens and a failure to recruit cells to sites of infection. More importantly, these observations suggest that the leucocytes of patients fail to extravasate and are retained in the vasculature by a failure to adhere to endothelial cells, giving the name of leucocyte adhesion deficiency (LAD) to this condition. Characterization of the proteins missing from the patients' leucocytes have confirmed their role in adhesion to endothelium and in other adhesion events.

LAD is caused by a failure of leucocytes to express three related proteins, LFA-1, CR3 and p150,95 of the integrin family of cell-cell and cell-matrix proteins (90). Each of these cell-surface glycoproteins consists of an $\alpha_1\beta_1$

dimer composed of a 150,-190,000 M_r α chain and a 95,000 M_r β chain. The β chain is identical in each of these three proteins (77) and has been given the designation CD18 by the International Workshop on Leucocyte Antigen Differentiation. The α chains are structurally and antigenically distinct (113). The α chains of LFA-1, CR3 and p150,95 are termed CD11a, CD11b and CD11c, respectively, and CR3 is referred to as CD11b/CD18.

LAD patients are deficient in all three of these proteins because of an inherited defect in the β chain (90). Cells from LAD patients do synthesize normal precursors of the α chains, but these precursors are very rapidly degraded and do not appear on the cell surface. Patients have recurring infections, often fatal in childhood unless they are corrected by bone marrow transplantation. In a study by Ross *et al.* (73), children with LAD had recurrent skin infections with *Staphylococcus epidermidis* but not yeast suggesting the role of CR3 and LFA-1 in the phagocytosis of unopsonized bacteria.

Lymphocytes express abundant LFA-1, but neither CR3 or p150,95. PMN express abundant CR3, whereas LFA-1 and p150,95 are minor but easily detectable components. Macrophages express large amounts of all three of these proteins. Expression of these proteins appears to be restricted to leucocytes; they have not been found on other mammalian cell types.

The first molecule shown to be deficient from LAD patients (6) had been previously identified as a receptor for a cell-bound fragment of complement

(114) and was named CR3 (complement receptor type three). CR3 has also been referred to as Mac-1 and Mo1 because it was the first phagocyte-specific antigen identified by monoclonal antibodies in mouse and man, respectively. CR3 functions as an opsonic receptor, and promotes the binding of C3bi-coated cells and particles by monocytes, macrophages and PMN (112, 117). In appropriately stimulated cells, binding is followed by phagocytosis of the particle but does not induce reactive oxygen metabolites (21). Therefore, CR3 provides a safe way for entry into professional phagocytes, compared to internalization via the Fc receptor. Most intracellular pathogens except viruses, have been shown to use CR3 for entry into mononuclear phagocytes. Examples are *Legionella pneumophila* (63), *Mycobacterium tuberculosis* (64), *Leishmania* sp. (98), and *Histoplasma capsulatum* (12).

CR3 requires relatively high concentrations of divalent cations (about 0.5 mM Ca^{2+} and Mg^{2+}) in order to interact effectively with ligand (112). Integrin α-subunits have three or four tandem repeats of a putative divalent cation-binding motif, and have been shown to require Ca^{2+} or Mg^{2+} for function (91). This behavior contrasts with that of other opsonic receptors (CR1 and FcR) which do not require divalent cations for binding activity. In addition, the binding capacity of CR3 is temperature-dependent, and is absent in cells held at 0°C (116), again distinguishing CR3 from other opsonic receptors (CR1 and FcR) which bind well at 0°C.

In the presence of serum, most pathogens are taken up via C3bi

deposition on their surface. CR3 does not recognize either the precursor C3b, or the product of further cleavage C3dg (14, 112, 72). Binding of monomeric or dimeric C3bi to CR3 has not been reported, presumably because of low binding affinity. Studies using sheep erythrocytes coated with 10^4 to 10^5 C3bi per cell demonstrate avid binding to CR3-bearing cells presumably because of multivalent interaction with the phagocytes. By analogy, it is likely that LFA-1 and p150,95 also bind their targets with low affinity and that adhesion events mediated by these receptors are driven by multivalent interactions. A functional consequence of low affinity binding is the potential to reverse adhesion and promote detachment.

CR3 has two distinct binding sites. One is located on the α chain and binds ligands such as C3bi, containing the Arg-Gly-Asp (RGD) sequence. In the absence of C3bi, microorganisms expressing a surface protein containing the RGD sequence could bind to CR3. For example, *Bordetella pertussis*, a gram-negative coccobacillus and the causative agent of whooping cough, has been shown to adhere to human macrophages *in vitro*. The interaction between macrophage CR3 and *B. pertussis* filamentous hemagglutinin (FHA) involves recognition of the RGD sequence at positions 1097-1099 in FHA (68).

The other binding site on the α chain of CR3 has a lectin-like character and, for example, recognizes lipopolysaccharide (LPS) located on the outer leaflet of the outer membrane of *Escherichia coli* (103, 116). *Histoplasma capsulatum* binds directly to CR3 via the lectin-like binding site (94). In

addition, an abundant surface glycolipid, promastigote lipophosphoglycan (LPG), from *Leishmania* has been shown to bind to the 'lectin-like-LPS' binding site on the α chain of CR3 (98, 77). It is possible that LPS, LPG and *H. capsulatum* display a common structure, such as a sugar phosphate, that can be recognized by CR3 (116).

Lipoteichoic acid as bacterial ligand for CR3

Group B streptococci contain membrane teichoic acid, lipoteichoic acid (LTA), which is known to mediate the adherence of GBS to human embryonic, fetal and adult epithelial cells (55). The membrane teichoic acids found in gram-positive bacteria exhibit a rather uniform structure: polyglycerol phosphate polymers with D-alanine and saccharides as constituents. (107). The hydrophobic end of the molecule interacts with the lipid bilayer of the membrane in combination with glycolipids or phospholipids. The hydrophilic chain of the teichoic acid, located on the outer surface of the membrane, extends into the space between the membrane and cell wall. Electron microscopy of bacteria using ferritin-labeled antibodies to LTA have shown that the polymer extends from the outer surface of the membrane through the cell wall and beyond the outer boundary of the cell into the external environment. The length of the membrane teichoic acid chain corresponds to the thickness of the cell wall as observed by electron microscopy.

Lipoteichoic acid from group B streptococci consists of a glycerol phosphate chain attached to a terminal lipid residue, identical in character to a free glycolipid of the cytoplasmic membrane. The glycerol phosphate backbone is substituted with glucose (55). Results from the study of Nealon and Mattingly (55) indicate that the binding mechanism of LTA from GBS to human embryonic and fetal cells is temperature dependent and is influenced by both the lipid moiety and the glycerol phosphate backbone. The binding of LTA appears to involve a hydrophobic interaction which is dependent on the membrane fluidity of the human cells as well as a hydrophilic interaction of the glycerol phosphate backbone with specific receptor sites on the human cells. The structural similiarities of the sugar phosphate part of the LTA molecule with that of both LPG and LPS, which are known to be bacterial ligands for CR3, suggest a possible role for LTA in the adherence of GBS to macrophages.

CHAPTER 1

A QUANTITATIVE METHOD FOR MEASURING THE ADHERENCE OF GROUP B STREPTOCOCCI TO MURINE PERITONEAL EXUDATE MACROPHAGES

Abstract

I have developed a solid phase, direct binding, enzyme-linked immunosorbent assay (ELISA) to detect and quantify the adherence of group B streptococci to murine macrophages. The assay correlated well with direct microscopic quantification of adherence. As few as 3.8×10^4 bacteria per assay well or less than 1 bacterium per macrophage could be detected. This assay is both quantitative and selective, and is readily adaptable for multiple sample analysis. It provides a valuable alternative to visual detection of bacterial adherence.

Introduction

The group B streptococcus (GBS) is the single most common agent associated with bacteremia and meningitis during the neonatal period (75). Polymorphonuclear neutrophils are known to phagocytose and destroy these bacteria, but this requires opsonization with either specific antibody or

complement (88). I have been examining the possibility that in individuals such as neonates, for whom serum opsonins may not yet be fully functional, macrophages serve to recognize and destroy potential pathogens in the absence of serum factors. As part of that study I required an *in vitro* binding assay for measuring the adherence of GBS to host defense cells.

My initial studies used the binding assay developed by Glass *et al.* (27), which involves the direct microscopic examination of stained cells. Although a valuable assay, it suffers from several limitations. The procedure is labor-intensive, the data are subjectively derived, and the assay lacks good reproducibility. Other protocols for measuring bacterial attachment to eukaryotic cells have been developed, including ones using fluorescence-activated cell sorting (61), fluorescence microscopy (59), radiolabeled bacteria (104), or electronic particle counters (31), but none provides the composite attributes of repeatability, versatility, and low cost I sought.

Ofek *et al.* (59) reported on a ELISA-based system for determining the adherence of bacteria to enterocytes or oral epithelial cells. Subsequently Athamna and Ofek (7) applied this approach to quantify the adherence of *Klebsiella pneumoniae* to mouse peritoneal macrophages. Here I report on an adaptation of this technique for quantifying adherence of a gram-positive bacterium to phagocytic cells.

Materials and methods

Bacteria Type II group B streptococci, strain 18RS21 (44), kindly provided by Dr. Dennis Kasper, Harvard Medical School, Boston, MA, were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) at 37°C to mid-log phase under static conditions. The bacteria were harvested by centrifugation and were washed in Dulbecco's phosphate-buffered saline (DPBS). Stock suspensions of GBS were stored at -70°C in DPBS containing 8% dimethyl-sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO). Before use in each assay frozen aliquots were thawed, washed with DPBS, and adjusted to the appropriate density. The concentration of bacteria in the suspension was determined by direct counts in a Petroff-Hausser chamber (C. A. Hausser and Son, Philadelphia, PA).

Macrophages Peritoneal exudate macrophages (pMø) from 8-12-week-old female BALB/c mice (originally obtained from Charles River Laboratories, Wilmington, MA and subsequently bred at our institution) were elicited by intraperitoneal injections of 2 ml of Brewer thioglycollate (Difco Laboratories). After 3 days the pMø were harvested by lavage using 10 ml of cold Medium 199 with Earle's salts (M199; Gibco Laboratories, Grand Island, NY). The pMø were washed twice by centrifugation at 100 x *g*, using cold M199 and resuspended in M199 to a concentration of 10^6 cells/ml.

Antibody Rabbit antiserum against the cell wall polysaccharide of type II GBS was kindly provided by Dr. Dennis Kasper.

Cell lines The WISH (ATCC CCL 25, Rockville, MD) and L929 (ATCC CCL1) cell lines were used as controls for bacterial attachment. Both cell lines were maintained in log phase using Minimal Essential Medium (MEM) with Earle's salts containing 15% fetal bovine serum (Hyclone Laboratories, Logan UT), 2 mM L-glutamine (Gibco), and 100 μM non-essential amino acids (Gibco) at 37°C with 5% CO_2, 95% air. The cell lines were harvested using 0.05% trypsin-EDTA (Gibco) and resuspended in M199 at a concentration of 10^6 cells/ml.

Immobilization of cells onto the microtitration plate Two-hundred microliters of pMø or of control cell lines (10^6 cells/ml) were distributed to the wells of a 16-chamber glass slide (Lab-Tek, Nunc, Inc., Naperville, IL). The cells were sedimented to the bottom of the plate by centrifugation at 100 x *g* for 5 min (Beckman model TJ-6; with micro-plate carrier for the TH-4 rotor), then allowed to attach for 3 h at 37°C. The cell monolayers were washed twice with DPBS to remove unattached cells.

Microscopic assay for measuring GBS binding to pMø I developed a modification of the original procedure (27). Suspensions of GBS in 100 μl of DPBS, at concentrations of 10^9, 2×10^8, and 4×10^7 bacteria/ml, were added to the pMø

and control cells in duplicate. After incubation at 37°C for 1 h the wells were washed 4 times with DPBS to remove nonadherent bacteria. The chambers were then removed and the slide air-dried, fixed in methanol, and stained with a modified Wright's stain (Leukostat, Fisher Scientific, Pittsburgh, PA). Approximately 200 pMø were examined per well for bacterial adherence. Those with 2 or more (27) or 5 or more bacteria attached were scored as positive.

ELISA Suspensions of GBS in 100 µl of DPBS, at concentrations of 10^9, 2×10^8, and 4×10^7 bacteria/ml were added to the macrophage and control cells in quadruplicate. After incubation at 37°C for 1 h the wells were washed four times with DPBS to remove nonadherent bacteria. The plates were air-dried and fixed with methanol for 10 min. Bacteria extracellularly attached to macrophages or control cells were quantified by ELISA.

The choice of a control for bacterial adherence is crucial. We attempted to use empty plastic wells or wells coated with protein blocking agents such as bovine serum albumin, gelatin, or hemoglobin (59) as negative controls. In all cases I found a high degree of bacterial adherence to the empty wells or protein-coated wells when compared with bacterial attachment to pMø. For this reason I chose to compare bacterial attachment to two types of easily obtainable, non-phagocytic cell lines with that of the macrophage. By using either the epithelial-like WISH cell line or the fibroblast-like L929 cell line as

controls for non-specific attachment of GBS to the wells of the assay chamber, I obtained low background ELISA readings in these wells, compared with ELISA values for GBS adhering to pMø.

The procedure of Athamna and Ofek (7) was modified for use with our system. To each well, containing methanol-fixed cells, was added 200 µl of 20 mM phosphate, 0.15 M NaCl (PBS), pH 7.2, containing 1% gelatin (Bio-Rad Laboratories, Richmond, CA), to block nonspecific binding of antibody, and 10 µg/ml of goat immunoglobulin G (Organon Teknika Corp., Durham, NC), to block the Fc receptors on the macrophages. After incubation for 1 h at 37°C, the monolayers were washed three times with PBS containing 0.05% Tween-20 (Bio-Rad; PBS-Tween), followed by the addition of 100 µl/well of specific anti-GBS serum diluted 1:500 in PBS containing 1% gelatin and 0.1% Tween-20 for 1 h at 37°C. The plates were washed three times with PBS–Tween, followed by the addition of 100 µl per well of horseradish peroxidase-labeled, affinity-purified anti-rabbit immunoglobulin G (Organon Teknika), diluted 1:5000 in PBS–Tween + gelatin for 1 h at 37°C. After three washes with PBS–Tween, 200 µl of the substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB; Sigma) in acetate buffer was added to each well. Since TMB is poorly soluble in aqueous solution, it was first dissolved in DMSO to a final concentration of 42 mM and 1 ml of this TMB-DMSO solution was added dropwise with gentle shaking to 100 ml of 0.1 M sodium acetate-citric acid buffer, pH 4.9. Just before use, 14.7 µl of 30% hydrogen peroxide was added to the acetate buffer contain-

ing TMB for a final concentration of 1.3 mM H_2O_2. The blue color was allowed to develop for 15 min at room temperature and the enzyme reaction was stopped with the addition of 50 µl of 2.0 M H_2SO_4 to each well. The absorbance was read at 450 nm (A_{450}) with an ELISA plate reader (Whittaker M.A. Bioproducts, Inc., Walkerville, MD). The following control systems were included: [1] blank wells (no bacteria or eukaryotic cells), [2] wells with macrophages only (no bacteria), and [3] wells with control cells (no bacteria). These controls were included to ensure that the eukaryotic cells did not cross-react with the first antibody and to detect any nonspecific binding of the antibodies.

Determination of the number of bacteria per monolayer A standard curve made for each test served to estimate the number of bacteria per monolayer. For this purpose GBS of known concentration in 100 µl of distilled water was placed in the wells of a microtitration plate and allowed to dry overnight in a desiccator, followed by fixation with methanol for 10 min. An ELISA was performed on the immobilized bacteria as described above. The ELISA values, at A_{450}, were plotted as a function of the number of bacteria in each well. The curve obtained was used to calculate the number of bacteria attached to the pMø or cell line monolayer from the ELISA values obtained in the test experiment.

The standard curve was adjusted for loss of dried bacteria due to washing

following the procedure of Athamna and Ofek (7). GBS were grown in tryptic soy broth (Difco) containing 1.5 μCi of 5-[^{125}I]iodo-2'-deoxyuridine (NEN-Dupont, Boston, MA)/ml to mid-log phase at 37°C under static conditions. The radiolabeled bacteria were harvested by centrifugation and washed free of excess radioactivity with DPBS. The bacteria were adjusted as above to the desired concentration and the radioactivity associated with the cells was determined using a gamma counter (Gamma 5500, Beckman Instruments, Inc., Fullerton, CA). The radiolabeled bacteria contained 3800 cpm/10^7 bacteria. The bacterial suspension was diluted in distilled water, dried, and fixed onto two sets of flat-bottomed EIA/RIA Strip-Plate-8 (Costar Corp., Cambridge, MA). One of the sets was washed 9 times with PBS–Tween to duplicate ELISA washes and the radioactivity of the individual wells of the two sets was determined.

Determination of the number of pMø per well This determination was based on the selective staining of the pMø nuclei with methylene blue, followed by extraction of the stain (10). Known concentrations of pMø in 100 μl of M199 were sedimented in the wells of a microtitration plate, air-dried, and fixed with methanol. These monolayers were stained with 100 μl of 1% methylene blue solution/well for 10 min, followed by washing with boric acid buffer (0.1 M boric acid, 0.1 M KCl, pH 8.6). The stain was extracted by adding 0.1 N HCl and the recovered solution read at A_{620} nm in the ELISA plate

reader. The A_{620} values of the extracted stain were plotted as a function of the number of pMø in each well to obtain a standard curve. This curve was used to estimate the number of pMø in each experimental well after extracting and reading the methylene blue stain from each test monolayer.

A standard curve made for each test served to derive the number of pMø per monolayer. After determination of the number of bacteria in the experimental assays was completed, the same monolayers were washed and stained to quantify the number of pMø, using the standard curve previously derived.

Results

Standard curves for adherence of GBS and pMø In order to quantify bacterial adherence using the ELISA system, it was first necessary to construct standard curves to determine the number of bacteria and of macrophages in each system. Serially diluted suspensions of GBS, dried and fixed on microtitration plates, were reacted with the ELISA reagents. As shown in Fig. 1, a linear relationship between the number of immobilized bacteria (expressed logarithmically) and the ELISA values was obtained over the range of 6×10^4 to 6×10^6 bacteria/well. To determine whether significant numbers of bacteria were lost during washing, surface-labeled (^{125}I) bacteria were dried and fixed to the bottom of microtitration plate wells. One series was washed nine times while the others remained unwashed. The amount of remaining radioactivity was

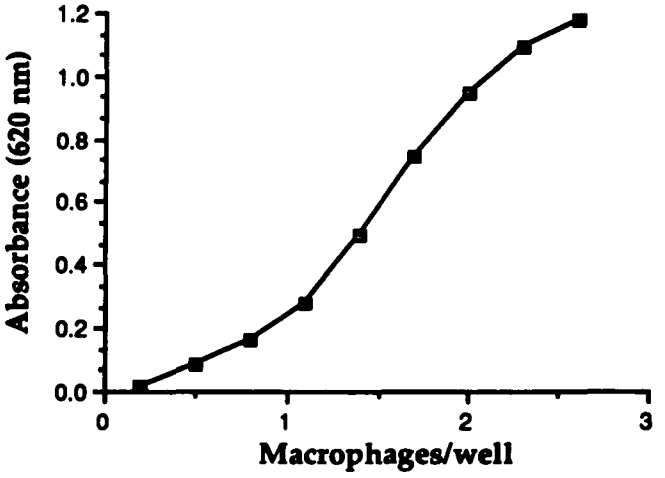

Fig. 1. Standard curve for the determination of numbers of bacteria per well. ELISA values (A_{450}) as a function of increasing numbers of dried, immobilized streptococci

determined for each sample. The data, depicted in Table I, indicate there was negligible loss of radioactivity, and hence of bacteria, due to washing over the concentration range of 2×10^3 to 2×10^8 bacteria added.

The number of adherent pMø was estimated by staining with methylene blue and quantifying the extracted dye. As shown in Fig. 2, the A_{620} values of the extracted methylene blue were linear over the range of 10^5 to 10^6 pMø/well. These standard curves were made in each experiment and used to determine the number of adherent bacteria per pMø, which was calculated by dividing the total number of adherent bacteria by the total number of pMø/well.

Adherence assays measured by ELISA The number of pMø or control cells added to each well of the assay plate was 2×10^5. Approximately 1×10^5 pMø were immobilized per well, as determined from the pMø standard curve. This number of cells formed a complete monolayer covering the surface area of the well. By visual inspection the WISH and L929 cells also completely covered the surface area of the well. Thus, any differences in ELISA values between wells containing immobilized pMø and those with control cells is a reflection of specific bacterial adherence to the pMø.

The adherence of GBS to pMø was dose-dependent over the concentration range of bacteria added, namely 4×10^6 to 1×10^8/ well. Adherence of less than

TABLE 1

EFFECT OF WASHING ON THE ADHERENCE OF BACTERIA

Use of ^{125}I-labeled GBS

No. of bacteria added	CPM for non-washed[a]	CPM for washed[a]	Percentage remaining after washing
2×10^8	36403±353	35687±477	98
4×10^7	6990±116	6848±65	98
8×10^6	1372±15	1353±8	99
1.6×10^6	301±13	323±12	107
3.2×10^5	106±11	104±19	98
6.4×10^4	57±8	58±13	102
1.3×10^4	43±7	50±16	116
2.6×10^3	49±5	54±3	110

[a] Mean ± S.E. (n = 4)

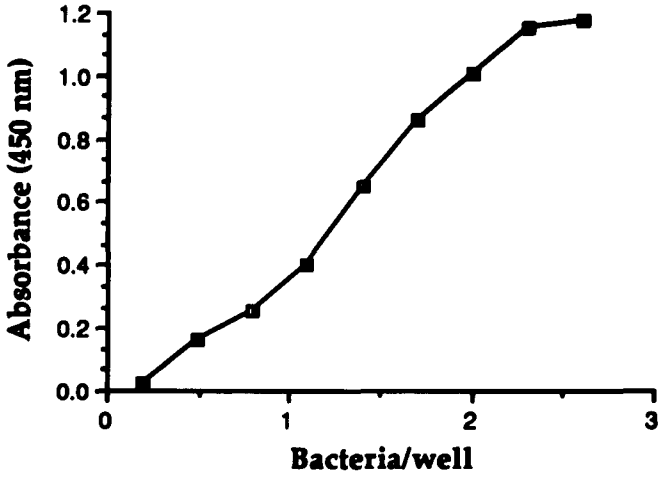

Fig. 2. Standard curve for the determination of numbers of macrophages per well. Values of extracted methylene blue stain (A_{620}) as a function of number of sedimented and dried peritoneal macrophages.

one bacterium per cell was detected by the ELISA technique (Table 2). To confirm that ELISA readings corresponded to bacterial adherence to pMø, the bottoms of the wells were cut off and mounted onto a glass microscope slide, and the number of adherent bacteria per pMø or control cell was counted microscopically. There was a good correlation between the ELISA readings for adherence and the values obtained by direct microscopic counts.

Comparable studies performed with non-phagocytic cell lines revealed significantly lower values for microbial adherence. Even at the highest dosage tested (1×10^8 bacteria added), the binding ratios were at least ten-fold lower than those for pMø.

Visual assay for detecting microbial attachment Standard adherence assays were performed by a modification of the technique developed by Glass *et al.* (27). For each assay system two values were determined: the number of pMø with ≥ 2 bacteria attached and the number of pMø with ≥ 5 bacteria attached. The former is the value used in the original description of this protocol, while the latter is a more rigorous criterion used in our laboratory.

The results of these assays are summarized in Table 3. As expected, there was a positive correlation between the number of bacteria added and the percentage of pMø exhibiting adherence. This was true regardless of which binding criterion was used. At the higher level of GBS added (1×10^8) virtually all the pMø bound at least 2 bacteria; however, only one-third of

TABLE 2

ATTACHMENT OF GROUP B STREPTOCOCCI TO MACROPHAGES AND CONTROL CELL LINES

Number of bacteria per well is based on ELISA values.

Cells	GBS added/well	Ratio of added GBS/Mø[a]	Mean no. (± S. E.) of GBS bound per Mø[b]
pMø	1×10^8	1000:1	8.5 ± 1.7
	2×10^7	200:1	1.5 ± 0.4
	4×10^6	40:1	0.38± 0.03
L929	1×10^8	1000:1	0.67± 0.10
	2×10^7	200:1	0.27± 0.06
	4×10^6	40:1	0.13± 0.04
WISH	1×10^8	1000:1	0.53± 0.06
	2×10^7	200:1	0.22± 0.04
	4×10^6	40:1	0.15 ± 0.01

[a] ~10^5 Mø/well

[b] n = 12

TABLE 3

ATTACHMENT OF GROUP B STREPTOCOCCI TO MACROPHAGES AND CONTROL CELL LINES

Numbers are based on visual assessment by direct microscopic counting

Cells	GBS added	Percentage with ≥ 2 bacteria/cell[a]	Percentage with ≥ 5 bacteria/cell[a]
pMø	1×10^8	96 ± 1.2	33 ± 3.5
	2×10^7	82 ± 1.7	9.7± 2.1
	4×10^6	10 ± 2.0	1.9± 0.8
L929	1×10^8	7.6± 1.1	2.7± 0.42
	2×10^7	1.9± 0.02	0.13 ± 0.02
	4×10^6	0.40 ± 0.04	0.05± 0.02
WISH	1×10^8	6.0 ± 0.57	2.3± 0.4
	2×10^7	1.6 ± 0.26	0.09 ± 0.02
	4×10^6	0.34 ± 0.05	0.05 ± 0.03

[a] Mean ± S. E. (n = 6)

them scored positive when the criterion was ≥ 5 bacteria bound per cell. Comparable studies with non-phagocytic cell lines revealed adherence values 10 times lower for each concentration of GBS tested, confirming that the adherence was not a general eukaryotic cell function.

As shown in Fig. 3, there was good correlation between the ELISA readings for adherence and the values obtained by direct microscopic counts.

Discussion

Phagocytosis of microorganisms is an important host defense among members of the animal kingdom. In order for phagocytosis to be initiated there must be contact between the engulfing cell and the potential pathogen. Assuming that these are not chance encounters there must be some form of recognition between these two participants. In the case of non-opsonin-mediated phagocytosis the ability to phagocytose microorganisms must be due to inherent properties of the host cell, most likely structures located on the plasma membrane.

As part of our studies to identify the receptor on the mouse pMø responsible for recognition of GBS I sought a relatively inexpensive binding assay that could be readily performed and would give reproducible results. An enzyme-linked assay has the potential to provide objective data and to allow simultaneous testing of multiple variables. Athamna and Ofek (7)

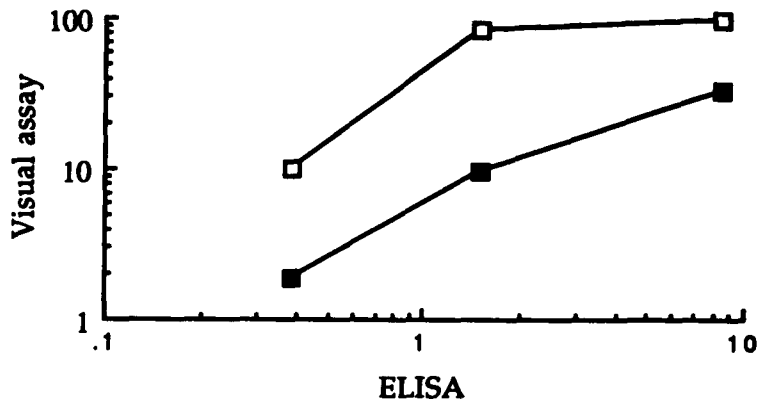

Fig. 3. Comparison of bacterial binding obtained from the direct visual assay and from ELISA. Plot with open squares relates results from visual assay (Mø binding ≥ 2 bacteria) with ELISA data (number of bacteria bound per Mø). Plot with closed squares depicts a comparable relationship between visual assay (Mø binding ≥ 5 bacteria) and ELISA data.

developed an ELISA-based adherence assay using *K. pneumoniae* and mouse pMø. My studies indicate that this assay can be modified to detect binding of GBS to pMø.

In this system bacteria are allowed to adhere to monolayers of pMø and their presence is both detected and quantified by an ELISA, using specific antibody to the bacteria as the primary antibody. To calculate binding ratios it was first necessary to verify the numbers of each cell type in our assay chambers. Standard curves plotting the numbers of bacteria per well vs. absorbance at 450 nm (to detect the product formed by horseradish peroxidase in the antibody-enzyme conjugate; Fig. 1) and the number of pMø per well vs. absorbance at 620 nm (to detect the methylene blue eluted from stained pMø; Fig. 2) each yielded sigmoidal dose-response curves with linear mid-sections.

Using this approach determined the binding ratios for GBS and murine pMø. As shown in Table 2, the data obtained are highly replicable. At high ratios of added bacteria to pMø (1000:1) our system detected 7-10 bacteria per pMø, whereas at a lower ratio (200:1) there were 1-2 bacteria detected per pMø. Under comparable conditions the control eukaryotic cells consistently exhibited bacterial binding ratios of <1 per cell.

Data presented in Table 3 provide information obtained from bacterial binding studies using direct microscopic counting. As with the ELISA-based system, results from the direct counting assays also yielded a positive dose-response effect. Similarly, the control cells gave consistently low binding

values.

Although the ELISA-based system for quantifying bacterial binding to eukaryotic cells provides some obvious advantages over direct visual assay, each system is, in fact, assessing a somewhat different aspect of adherence. Using ELISA we can obtain information on a population of Mø, but we cannot determine the distribution of the bound and detected bacteria over this population. That is, we can calculate an average binding ratio, *e.g.*, 8.5 bacteria per pMø, but we cannot ascertain the binding properties of individual Mø; for this the visual assay system remains the definitive procedure.

Nonetheless the use of an ELISA-based approach to measure bacterial adherence provides a valuable tool for screening various assay conditions or treatments to determine their effect on binding , since many variables can be simultaneously tested. The ability of this system to detect as few as 3.8×10^4 GBS compares favorably with the data of Athamna and Ofek (7) in which they detected 5×10^4 *K. pneumoniae*. These studies indicate that an ELISA-based system has broad application in studies on microbial phagocytosis.

CHAPTER 2

CHARACTERIZATION OF THE MURINE MACROPHAGE RECEPTOR FOR GROUP B STREPTOCOCCI

Abstract

I have examined the role of the galactose receptor for the non-opsonin mediated adherence of group B streptococci (GBS) to murine peritoneal macrophages (Mø). The effects of sugars and neoglycoconjugate proteins of bovine serum albumin (BSA) that are known to bind to the lectin-like receptor on the inhibition of GBS binding to peritoneal macrophages was investigated. These studies required stringent conditions, both a visual assay and enzyme-linked immunosorbent assay (ELISA), for quantifying binding and the subsequent inhibition of binding. Using these assay conditions I observed no inhibition of binding by monosaccharides, including methyl-β-galactoside, at concentrations as high as 200 mM or the neoglycoconjugate protein, galactose-BSA, at 2 mg/ml (~29 μM galactose). To increase the exposure of capsular galactose, terminal sialic acid was removed by treating GBS with neuraminidase from *V. cholerae*. Dose-dependent binding of

neuraminidase-treated and untreated GBS types Ib, II and III was similiar. In addition, the binding profile was similar for wild-type and isogenic strains of type III GBS having no capsule or no capsular sialic acid. Galactose or galactose-BSA failed to inhibit binding of any of these strains. These results suggest that there is no involvement of the galactose lectin-like receptor on murine peritoneal macrophages with the binding of GBS in the absence of serum opsonins.

Introduction

Group B streptococci (GBS) are a major cause of neonatal meningitis and septicemia in the United States (80) and in post-partum infections. Increasingly they are being recognized as an important pathogen in non-pregnant adult humans as well (81). Phagocytosis of these organisms followed by intracellular killing is considered to be the major host defense in mammals and serum opsonins, *e.g.*, anti-capsule antibody and complement, have been shown to play a major role in effecting endocytosis (13).

GBS also bind directly to mammalian macrophages in the absence of serum opsonins (see Chapter 1; ref. 25). Non-opsonin-mediated phagocytosis can be achieved by membrane-associated lectins on the macrophage reacting with complementary sugar residues on the microbial surface (106, 82).

These bacteria are typically encapsulated and the structures of these

polysaccharide capsules form the basis of serotype designations (39, 38, 41. 40). Currently four serotypes, Ia, Ib, II, and III, are recognized; their basic structure is shown in Fig. 1 (p. 9, Chap. 1; ref. 44). The backbone in each case is composed of a repeating unit of 2 to 5 monosaccharides, while the side chain β-D-galactose is either exposed or penultimate to a terminal sialic acid.

My hypothesis, then, was that macrophage-derived lectins mediate the recognition and binding of GBS. The data reported here do not support this hypothesis.

Materials and Methods

Chemicals All reagents were obtained from Sigma Chemical Co., St. Louis, MO unless otherwise noted.

Bacteria Prototypic strains of GBS, representing two serotypes, originally obtained from the late Dr. Rebecca Lancefield, Rockefeller University, were provided by Dr. Dennis Kasper, Harvard Medical School. These strains are designated H36B (type Ib), 18RS21 (type II) (44). Two type III GBS strains M732 and COH31, originally clinical isolates from infants with meningitis (44), were also provided by D. Kasper. All strains were obtained as frozen suspensions of pure cultures, which were stored at -70°C. Each strain was streaked onto a 5% blood agar plate and incubated overnight at 37°C. A colony from each strain was used to inoculate 10 ml of Todd-Hewitt broth (Difco Laboratories,

Detroit MI) and this was incubated overnight to stationary phase. A 5-ml volume of each strain was used to inoculate one liter of Todd-Hewitt broth and this was incubated at 37°C to log phase under static conditions. The bacteria were harvested by centrifugation at 10,000 x*g* for 10 min and were washed three times in Dulbecco's phosphate-buffered saline (DPBS). Stock suspensions of GBS were adjusted to a density of 10^{10}/ml as determined by direct counts in a Petroff-Hausser chamber (C. Hausser and Son, Philadelphia, PA) and were stored at -70°C in DPBS containing 8% dimethylsulfoxide (DMSO). Before use in each assay, frozen aliquots from the original batch culture of each strain were thawed and washed with DPBS to insure continuity between experimental studies performed on different days.

Neuraminidase treatment of GBS Desialylation of GBS strains H36B, 18RS21, M732 and COH31 was done by adding 0.15 units of neuraminidase from *Vibrio cholerae*, (Sigma, Type II, specific activity: 10 units/mg protein using NAN-lactose) to 3 ml of bacterial suspension at 10^{10}/ml in 0.05 M sodium acetate buffer, pH 5.5, containing 154 mM NaCl and 9 mM $CaCl_2$ (27). After 1 h at 37°C with constant end-over-end rotation, the bacteria were washed three times with DPBS and stored in 8% DMSO as described above. Nondesialylated bacteria were treated likewise but without the addition of neuraminidase.

Isogenic strains of type III GBS COH31 Capsular mutants of GBS type III

strain COH31 obtained from D. Kasper (Harvard) were derived by Tn916 transposon mutagenesis. The mutant strain designated COH31-15 does not react with type III GBS antiserum from rabbits and has no visible extracellular capsule material when visualized by electron microscopy (45). Another mutant strain designated COH31-21 is lacking terminal sialic acid on the side chain, leaving βD-galactose as the terminal sugar.

Interaction of GBS with* Ricinus communis *agglutinin The availability of galactose residues on the surface of GBS was verified by the aggregation of a 1% suspension of each strain by the galactose-specific lectin from *Ricinus communis* (castor bean) at a concentration of 0.2 μg/ml. D(+)-Galactose, D(+)-glucose and methyl βD-galactopyranoside were tested at concentrations of 10 mg/ml, 1.0 mg/ml and 0.1 mg/ml for their ability to inhibit the aggregated GBS.

Detection of β-glucan receptor on Mø The β-glucan receptor found on Mø was used as a control system for the inhibition studies. *Saccharomyces cerevisiae* and a preparation of its cell wall as particulate zymosan have been shown to bind to Mø in the absence of serum opsonins (96). Zymosan particles consist almost exclusively of two types of carbohydrate polymers: β-glucans, which are the major constituents, and α-mannans (22). Macrophage binding of *S. cerevisiae* zymosan is inhibited most effectively by the soluble β-glucans purified from yeast extract or yeast glucan particles with 1,3- and/or

1,6-glycosidic linkages (37), whereas β-glucan from barley with 1,4-linkages are not (17, 18). Homopolysaccharides containing epimers of glucose, such as galactans and yeast α-mannans are not inhibitory even though they contain 1,3- and/or 1,6-linkages.

β-Glucan from *S. cerevisiae* inhibits binding through the β-glucan receptor (19). I have exploited this mechanism for macrophage binding to a surface structure of yeast as a positive control in our inhibition studies. A suspension of zymosan A from *Saccharomyces cerevisiae*, measuring 3-4 μm in diameter, was prepared by sonication using a pulsed cycle for 30 seconds, at output setting 4, 50% duty cycle (Heat Systems Ultrasonics, Inc. Plainview, NY) in DPBS. Soluble α-mannan and β-glucan from *S. cerevisiae* and β-glucan from barley were tested for their ability to inhibit binding of zymosan to Mø. The lowest concentration of each capable of yielding 50% inhibition of zymosan attachment to Mø at particle concentrations of 10^9/ml and 10^8/ml was determined by the visual assay.

Macrophages Peritoneal exudate macrophages (Mø) from 8 to 12 week-old female BALB/c mice were elicited by intraperitoneal injections of 2 ml of Brewer thioglycollate (Difco Laboratories). After 3 days the Mø were harvested by lavage using 10 ml of RPMI medium (Gibco Laboratories, Grand Island, NY). The Mø were washed twice by centrifugation at 100 x*g* for 10 min, using cold RPMI and resuspended in RPMI to a concentration of 10^6

cells per ml.

Antisera to GBS Group-specific and type-specific antisera to GBS, prepared in rabbits, was kindly provided by Dennis Kasper, Harvard Medical School.

Binding Assays Adherence of GBS to Mø was determined either by direct microscopic observation or by an ELISA-based system, using previously described methodology (see Chapter 1).

Inhibition studies Macrophage monolayers were pre-incubated with monosaccharides in DPBS for 30 min and then for 1 h in the presence of bacteria. The following monosaccharides were used at concentrations of 2, 20 and 200mM: D(+)-galactose, methyl α-D-galactoside, methyl β-D-galactoside, D(+)-glucose, methyl α-D-glucoside, methyl β-D-glucoside, D(+)-mannose, methyl α-D-mannoside, methyl α-L-fucose, *N*-acetyl-D-glucosamine (GluNAc) and *N*-acetyl-D-galactosamine (GalNAc). Neoglycoconjugates prepared by E·Y Laboratories, San Mateo, CA, from bovine serum albumin (BSA), containing 30 to 40 mole saccharide per mole BSA, were tested for their ability to inhibit binding of GBS to macrophages. Glucosylated-, galactosylated- and mannosylated-BSA were incubated with the Mø monolayers as described for the monosaccharide solutions at concentrations of 20, 200 and 2000 μg/ml. The concentrations of the monosaccharide were equivalent to approximately 11, 110 and 1100 μM, respectively.

Unconjugated BSA, Fraction V, was included as a negative control at the same protein concentrations.

Results

Effect of sugars as inhibitors of GBS adherence to macrophages A comparison was made between galactose and other monosaccharides as well as BSA-glycoconjugates for the inhibition of type II and III GBS adherence to peritoneal macrophages. Using the visual assay I found there was no significant inhibition of the binding of either serotype by galactose or any of the other sugars at concentrations $\leq$ 200mM (Table 1) or the neoglycoconjugates at concentrations $\leq$2mg/ml (Table 2). The specific binding of zymosan particles from *S. cerevisiae* to the β-glucan receptor on Mø was included as a positive control to verify that the visual assay can quantify receptor inhibition (Table 3). The data in Table 1 suggest that the mechanism for type II and III adherence was not due to the galactose receptor on the macrophage, since there was no difference in binding by serotype or inhibition of binding specifically by galactose.

Effect of desialylation of capsular GBS polysaccharide The binding of GBS to Mø may be due to more than one type of mechanism including the galactose receptor. However, the inability of galactose to inhibit binding could be due to the masking effect of terminal sialic acid. GBS types Ib, II and III were

TABLE 1

EFFECT OF MONOSACCHARIDES AS INHIBITORS OF GBS ADHERENCE TO Mø

Inhibitor[a]	Strain of GBS 18RS21	M732	COH31
PBS	100 ± 11[b]	100 ± 8	100 ± 12
D(+)glucose	62 ± 9[c]	55 ± 13	64 ± 12
Methyl-α-glucose	69 ± 13	45 ± 12	60 ± 6
Methyl-β-glucose	59 ± 7	58 ± 10	76 ± 8
D(+)galactose	69 ± 12	58 ± 8	68 ± 15
Methyl-α-galactose	64 ± 8	55 ± 14	76 ± 12
Methyl-β-galactose	64 ± 10	55 ± 11	68 ± 6
L(-)fucose	64 ± 6	58 ± 11	76 ± 9
D(+)mannose	76 ± 14	63 ± 9	80 ± 0
Methyl-α-mannose	71 ± 12	84 ± 14	90 ± 12
N-acetylglucosamine	69 ± 8	77 ± 7	96 ± 10
N-acetylgalactosamine	66 ± 8	68 ± 6	96 ± 10

[a] Inhibitors were used at concentrations of 200 mM

[b] Percentage binding ± S.E. (N=8)

[c] Analysis of variance for all systems (excluding PBS controls) indicated no statistically significant difference in the effects of the test sugars on bacterial adherence ($F = 2.113$; $p = 0.0785$)

TABLE 2.

EFFECT OF BSA-GLYCOCONJUGATES AS INHIBITORS OF GBS ADHERENCE TO Mø

Inhibitors[a]	Strains of GBS		
	18RS21	M732	COH31
PBS	100 ± 8[b]	100 ± 10	100 ± 15
Glucose–BSA	112 ± 18[c]	100 ± 8	96 ± 9
Galactose–BSA	115 ± 20	100 ± 10	115 ± 6
Mannose–BSA	112 ± 9	94 ± 12	104 ± 12
Glucose/galactose–BSA	112 ± 12	115 ± 13	133 ± 15
Glucose/mannose–BSA	106 ± 11	106 ± 15	136 ± 21
Galactose-mannose–BSA	115 ± 9	94 ± 7	133 ± 16

[a] Inhibitors were used at concentrations of 2 mg/ml.

[b] Percentage binding ± S.D. (N=8)

[c] Analysis of variance for all systems (excluding PBS controls) indicated no statistically significant difference in the effects of the test glycoconjugates on bacterial adherence ($F = 1.18$; $p = 0.38$)

TABLE 3

INHIBITION OF BINDING OF *SACCHAROMYCES CEREVISIAE* ZYMOSAN PARTICLES TO Mø

Inhibitor	Number of zymosan particles added	
	10^9/ml	10^8/ml
β-glucan (yeast)	0.5 mg/ml[a]	0.1 mg/ml
β-glucan (barley)	> 10 mg/ml	> 10 mg/ml
α-mannan (yeast)	> 10 mg/ml	> 10/mg/ml

[a] lowest concentration to yield 50% inhibition of binding. Representative data from visual binding assay.

treated with neuraminidase from *V. cholerae* to remove this sialic acid. I employed the lectin from *Ricinus communis* (castor bean) with a known specificity for β-D-galactose to determine if this residue was exposed following neuraminidase treatment. Table 4 represents results by slide agglutination for treated and untreated GBS. Agglutination of GBS by the castor bean lectin was specifically inhibited by D(+)galactose and methyl-β-galactoside, whereas D(+)glucose had no effect. Although desialylation by neuraminidase was shown to expose galactose residues, dose-dependent binding of neuraminidase-treated and untreated GBS type Ib, II and III to Mø as determined by ELISA is similar (Figure 1).

Comparison of type III isogenic strains for adherence to macrophages A further study comparing macrophage adherence of wild-type COH31 to isogenic strains of GBS type III, derived by Tn916 transposon mutagenesis and having no capsular sialic acid (COH31-21) or no capsule expressed (COH31-15) was performed by the visual assay. Table 5 represents data from three experiments in which the binding of all isogenic strains was similiar, about 35% and 12% for bacterial concentrations of 10^9/ml and 10^8/ml, respectively. As in previous studies 200 mM galactose was shown to have no inhibitory effect on the binding of any of the GBS isogenic strains to Mø, suggesting no involvement of the galactose lectin receptor.

TABLE 4

EFFECT OF MONOSACCHARIDES ON THE AGGLUTINATION OF NEURAMINIDASE-TREATED AND UNTREATED GBS BY *RICINUS COMMUNIS* AGGLUTININ

GBS strain	Treatment[b]	Inhibitor[a]			
		PBS	D(+)-gal	Meβ-gal	D(+)-glc
18RS21	–	+	<10[c]	<10	>10
	+	++	<1	<0.1	>10
M732	–	–	NT	NT	NT
	+	++	<1	<0.1	>10
COH31	–	–	NT	NT	NT
	+	++	<1	<0.1	>10
COH31-15	–	–	NT	NT	NT
COH31-21	–	++	<1	<0.1	>10

[a] inhibitors: PBS – control; D(+)-gal: D(+)-galactose; Meβ–gal: methyl-β-galactoside; D(+)-glc: D(+)-glucose

[b] +, neuraminidase-treated; –, untreated

[c] concentration that effected 50% inhibition of agglutination; NT, not tested

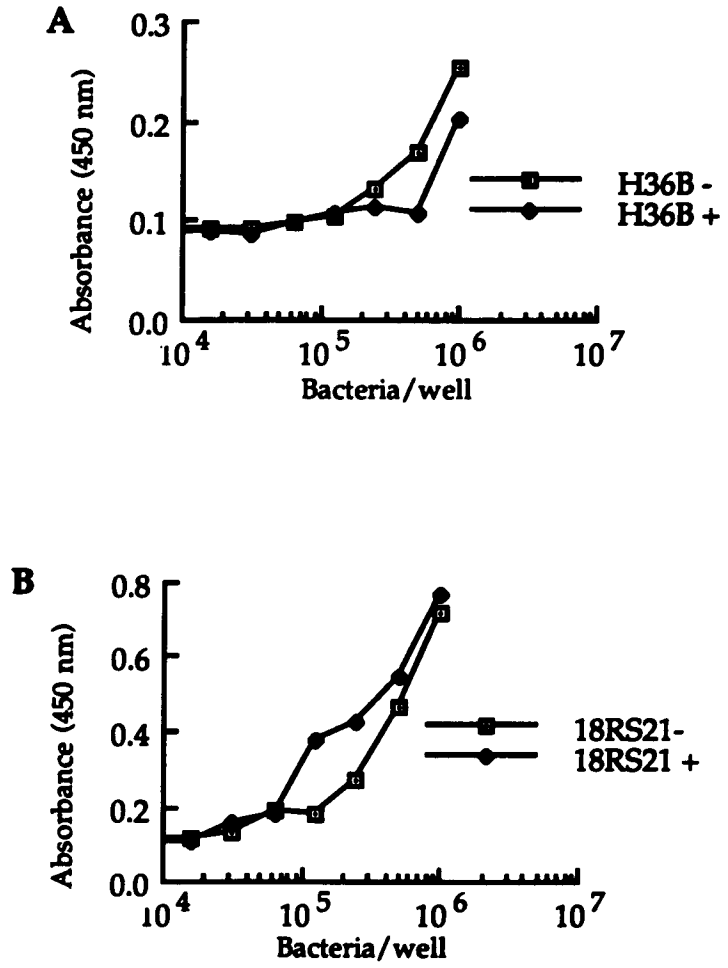

Fig. 1. Effect of neuraminidase treatment on adherence of four GBS strains to Mø. In each case (+) indicates treated system and (–), the untreated control.

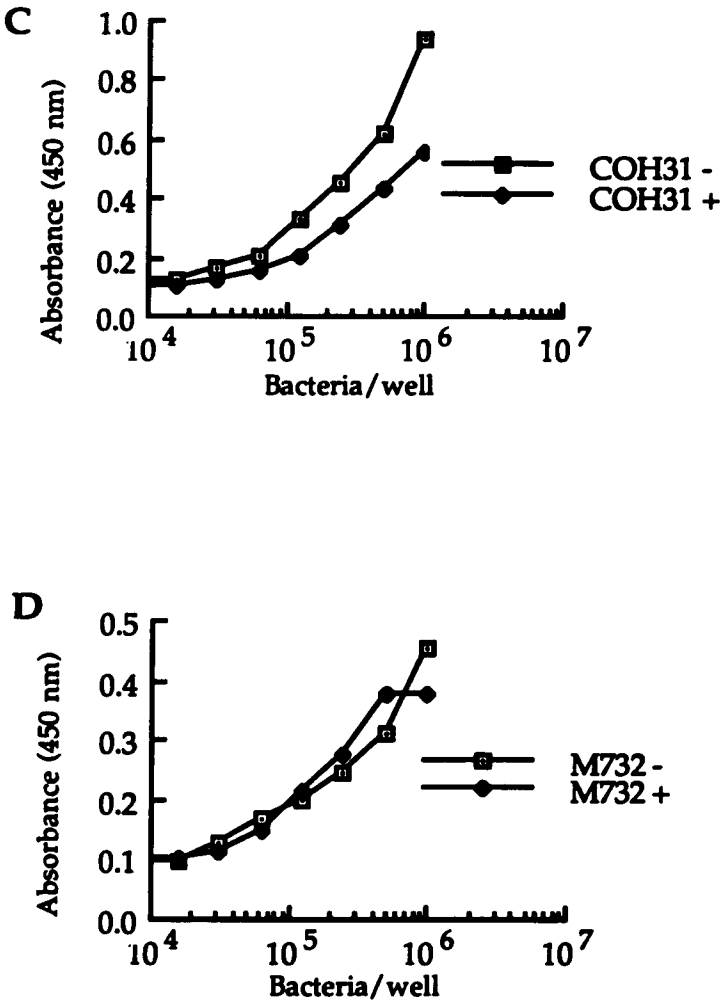

Fig. 1. (cont) Effect of neuraminidase treatment on adherence of four GBS strains to Mø.

TABLE 5

EFFECT OF D(+)-GALACTOSE ON THE ADHERENCE OF GBS TYPE III ISOGENIC STRAINS TO Mø

GBS strain	D(+)-gal added[a]	Bacteria/ml 10^9	Bacteria/ml 10^8
COH 31	–	30 ± 2[b]	10 ± 2
	+	33 ± 4	12 ± 1
COH 31-15	–	35 ± 5	9 ± 3
	+	30 ± 4	12 ± 1
COH 31-21	–	37 ± 3	13 ± 1
	+	39 ± 6	14 ± 3

[a] –, none added; +, 200 mM D(+)-galactose added

[b] percent binding ± S.D. (N = 6)

Discussion

Evidence from *in vitro* studies indicate that phagocytosis of bacteria occurs in opsonin-free media (20, 33, 43, 53, 69, 70, 71), while findings from *in vivo* studies show clearance of bacteria from the serum opsonin-deficient lungs by alveolar macrophages (28, 51) or from the blood by the reticuloendothelial system of animals depleted of complement (11). The molecular basis for non-opsonic recognition between bacteria and phagocytes is largely unknown.

Several investigators have postulated that the net surface charge or the hydrophobicity of particles or bacteria determines whether they can be bound by phagocytes (92, 102), but no direct evidence for these assumptions has been obtained. It is still possible that nonspecific or ill-defined surface properties may account for the recognition by phagocytes of some particles (*e.g.*, polystyrene beads). However, as pointed out by Griffin (33), recognition of most physiologically relevant particles must involve more specific mechanisms.

During the last decade, considerable evidence has accumulated showing that specific recognition by phagocytes may be accomplished by the interaction of carbohydrate-binding proteins, *e.g.*, lectins, in the cytoplasmic membrane of this defense cell with complementary sugars on the target cell (82, 83). This type of recognition, which also leads to phagocytosis, has been termed lectinophagocytosis (60). Macrophages have several cell-associated lectins that have been implicated in microbial recognition (30). A mannose-specific receptor

on human macrophages has been shown to recognize mannose-bearing pathogens and to mediate their internalization (24). The mannose/fucosyl receptor (MFR) recognizes *Leishmania* promastigotes and synthesis of reactive oxygen metabolites is triggered by adherence to this receptor (46). Human monocytes bear β-glucan receptors that react with zymosan particles (19), thus providing the cells with the ability to detect microorganisms bearing this glycan in the absence of serum factors. The clearance of *Klebsiella pneumoniae* from the lung may be due to a mannosyl/N-acetyl-D-glucosaminyl receptor found on alveolar macrophages (7).

Rat Kupffer cells *in vitro* strongly bind neuraminidase-treated rat erythrocytes but not untreated erythrocytes (49). Preincubation of the macrophages with D-galactose and related sugars inhibits cell adherence and suggests that the galactose-specific lectin receptor recognizes senescent erythrocytes (79).

In this study we attempted to show involvement of the galactose receptor for the non-opsonin-mediated adherence of GBS to peritoneal macrophages. My results, however, do not support this hypothesis. First, none of the monosaccharides or BSA-glycoconjugates acted as a specific inhibitor of type II and III GBS adherence to macrophages (Table 1,2). Second, neuraminidase-treated GBS, which were shown to have galactose exposed on their surface by agglutination with a galactose-specific lectin (Table 3), bound to peritoneal macrophages, but the binding could not be reduced by pre-exposure of the

macrophages to galactose (Table 4), again suggesting no involvement of the galactose receptor on the macrophage. Third, isogenic strains of type III GBS having no capsule or no sialic acid expressed, leaving galactose residues exposed, exhibited the same degree of attachment as wild-type type III to macrophages (Table 5).

Attachment with subsequent phagocytosis of microbial elements such as zymosan mediated by the β-glucan-type lectin on the surface on the surface of macrophages has been described by Czop *et al.* (19). This binding is inhibited most effectively by the soluble β-glucan from *S. cerevisiae* with 1,3 or 1,6 glycosidic linkages (37); β-glucan from barley with 1,4 linkages is not inhibitory (17, 18). Homopolymers containing epimers of glucose, such as galactans or yeast α-mannans are also non-inhibitory, even though they contain 1,3 or 1,6 linkages. My results (Table 3) confirmed the presence of the β-glucan-type receptor on the Mø. Additionally, this system served as a positive control for our inhibition studies.

Inhibition of blood clearance of neuraminidase-treated type I GBS, which express galactosyl residues on their surfaces, was shown to be strongly inhibited by galactosyl- but not by mannosyl- or fucosyl-BSA (65). In the same study the blood clearance of type II GBS could be inhibited by neither galactosyl- or mannosyl-BSA alone, whereas the presence of both neoglycoconjugates in the injected suspension significantly inhibited the blood clearance of streptococci. No such synergism was obtained in my experiments with type II and III GBS

adherence to murine peritoneal macrophages. These results suggest that lectins expressed on the surface of phagocytic cells in the liver are not present on peritoneal macrophages. However, in the same study (65) the adherence of type II GBS to Mø was inhibited by galactosy-, mannosyl- and glucosyl-BSA. As with blood clearance, the combination of galactosyl- and glucosyl- or mannosyl-BSA significantly increase the inhibition of attachment.

Differences in the results of my studies and those of Perry *et al.*(65) can not fully be explained by the difference in the type of phagocytic cell used (peritoneal macrophage vs. Kupffer) or the strain of mouse used (BALB/c vs. ICR) or the different strains of GBS. In my studies, the stringency of my conditions for detecting inhibition of adherence of GBS to Mø suggests another receptor or combination of multiple receptors for microorganisms that represents an efficient and potent defense mechanism for non-opsonin-mediated adherence. Possible candidates for these receptors may the macrophage integrins. The β_2 integrins CR3 and LFA-1 have been implicated in the phagocytic uptake of *Staphylococcus epidermidis* (96). Preliminary studies in our laboratory suggest that these leukocyte integrins are also the receptors involved in attachment of GBS.

CHAPTER 3

CHARACTERIZATION OF THE INTERACTION BETWEEN GROUP B STREPTOCOCCI AND THIOGLYCOLLATE-ELICITED PERITONEAL MACROPHAGES

Abstract

Macrophages have been shown by several investigators to recognize and bind group B streptococci (GBS) in the absence of serum opsonins. The mechanism for this binding has not been elucidated. I have shown this mechanism to involve a protein receptor on the macrophage since the binding of GBS is sensitive to trypsin treatment. Other characteristics of this binding, such as temperature dependence and a requirement for divalent cations suggest that the receptor involved is not the lectin-like galactose receptor. Treatment of the macrophages with 2-deoxy-D-glucose, at concentrations of 0.5 mM and 50mM, decreased GBS binding by 33% and 90%, respectively. Two-deoxy-D-glucose has been shown to inhibit Fc and complement receptor-mediated binding by mouse peritoneal macrophages. The function of receptors for the third component of complement (C3) to bind ligand-coated particles may be altered dramatically when macrophages

attach to surfaces coated with fibronectin (FN) or after macrophages are exposed to phorbol esters. Here I show that when phorbol dibutyrate (PDB) is present during the incubation of GBS and macrophages monolayer, binding doubles. In addition, when macrophages are allowed to attach to an FN-coated surface, in the presence or absence of PDB, binding of GBS also increases two-fold. Neutrophil elastase has been shownto cleave the C3b receptor, CR1, but not the C3bi receptor, CR3. When I treated the macrophage monolayers with neutrophil elastase, the binding of GBS was unchanged. These results suggest that the binding of GBS by peritoneal macrophages, in the absence of serum components, may be mediated by CR3.

Introduction

Phagocytic cells in higher animals are capable of discriminating between foreign material and self components. They are also able to distinguish between self and alterations to self, as in effete or damaged cellular components or aberrant self constituents, such as tumor cells (47). The recognition abilities that underlie this discriminating capacity must have evolved with the emergence of primitive species. Some form of self recognition mechanism is likely to have formed the basis of the abilities of colonial marine species such as *Porifera* to exclude unrelated organisms from inclusion in their colonies (105). In addition the cells lining the cavity of *Porifera* are

capable of capturing microorganisms present in the water drawn into the cavity. Phagocytosis also plays a role in the metamorphosis of insects in removing dead cells and disintegrated tissue (26). The role of invertebrate amebocytes in recognizing and engulfing foreign material is likely to be a development of these earlier, more primitive recognition mechanisms and begins to involve cooperation with soluble factors present in the hemolymph, acting as opsonins (67).

In vertebrates further specialization has occurred, involving cooperation between phagocytes and components of the immune system including antibodies, complement and lymphoid cells. The more primitive forms of phagocyte recognition appear, however, to have been retained and the cells appear to be able to recognize and bind foreign particulate material without the use of their more recently evolved receptors for the Fc component of the immunoglobulin molecule or the C3b component of the complement system (94).

I have observed nonopsonin-mediated binding of group B streptococci to murine peritoneal macrophages. Attempts to demonstrate adherence of exposed galactose of the bacterial capsule to the known macrophage membrane galactose receptor have been unsuccessful (see Chapter 2). In this study I have compared characteristics of the adherence of type III GBS to murine peritoneal macrophages with known characteristics of macrophage bacterial receptors in an attempt to identify whether a previously characterized mem-

brane receptor is responsible for this initial recognition.

Material and Methods

Chemicals All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Bacteria A culture of type III Group B streptococci, designated as strain COH31, was kindly provided by Dennis Kasper, Channing Laboratory (Harvard Medical School). This strain was originally isolated from an infant with meningitis (44). Capsular mutants of GBS type III strain COH31 were obtained from D. Kasper (Harvard) and are previously described (see Chapter 2). All strains were maintained, and prepared for the assay as previously described (see Chapter 2).

Macrophages Macrophages were prepared as previously described (see Chapter 2).

Assay of GBS adherence Monolayers of macrophages (prepared as previously described in Chapter 2) were layered with 0.2 ml of GBS at densities of 10^{10}/ml and 10^{9}/ml in DPBS and incubated at 37°C in the CO_2 chamber for 1 h. Bacteria were added and adherence quantified as previously described (see Chapter 1).

Treatment of macrophage monolayers with trypsin The procedure for trypsin treatment of macrophage monolayers was a modification of that devised by Czop *et al.* (16). Macrophages were layered with 0.25 ml analytical grade, bovine pancreatic trypsin (Boehringer Mannheim, Indianapolis, IN) having a specific activity, determined by the manufacturer, of 40 U/mg lyophilized material (25°C; benzoyl-L-arginine ethyl ester as substrate). The trypsin was diluted in RPMI containing an additional 5 mM $MgCl_2$, over the range of 4.0 μg/ml to 4.0 mg/ml, and the treated macrophages were incubated at 37°C for 30 min in the CO_2 chamber, and rinsed twice with RPMI. Each monolayer was then layered with 0.25 ml aprotinin (Trasylol ™, 200 U/mg lyophilizate trypsin inhibitor units, Boehringer Mannheim) at varying molar concentrations selected to be the equivalent of the previous trypsin concentration, incubated for 15 min at 37°C, rinsed four times with RPMI, and overlaid with wild-type COH31 and the two isogenic strains of GBS for detection of adherence. Treatment of macrophage monolayers with aprotinin alone, at any of the concentrations tested, followed by washing did not inhibit adherence.

Treatment of macrophage monolayers with neutrophil elastase Lyophilized human neutrophil elastase (Sigma) with a specific activity of 110 U/mg protein (determined and defined by the manufacturer as: one unit will release one nanomole of p-nitrophenol per second from N-t-BOC-L-alanine p-nitrophenyl ester at pH 6.5 at 37°C) was dissolved in sterile distilled water at

1 mg/ml and stored in small aliquots at -70°C. Macrophage monolayers were treated with 0.25 ml of neutrophil elastase at final concentrations of 30, 10 and 3 μg/ml in DPBS for 1 h at 37°C, using a modification of the method of Tosi *et al.* (100). The elastase was removed by washing the chambers individually four times with DPBS followed by the addition of GBS for assay of adherence. In addition, we examined the effect of elastase treatment on macrophage monolayers morphologically at 1000 X magnification.

Treatment of macrophage monolayers with 2-deoxy-D-glucose I used a modification of the method of Sung and Silverstein (97) to examine the effect of 2-deoxy-D-glucose treatment of macrophages on their ability to bind GBS. Briefly, macrophage monolayers were incubated with 500, 50, 5.0 and 0.5 mM 2-deoxy-D-glucose for 2 h at 37°C, followed by the addition of GBS to the monolayers for 1 h at 37°C, without removal of 2-deoxy-D-glucose, for assay of adherence. The morphological effect of 2-deoxy-D-glucose on macrophages without the addition of GBS was examined at 1000 X magnification.

Role of temperature and divalent cations on the adherence of GBS to macrophages Macrophage monolayers were incubated with GBS strain COH31 as described in the adherence assay at 37°C and at the additional temperatures of 20°C and 4°C to determine the effect of temperature on GBS adherence to Mø.

The effect of 0.5 mM $MgCl_2$ and $CaCl_2$ on this adherence was also tested using a modification of the method of Wright and Jong (116). Thioglycollate-

elicited peritoneal exudate cells were washed with Ca^{2+} and Mg^{2+}- free DPBS, then incubated for 3 h in the chamber-slide to prepare macrophage monolayers absent of divalent cations. GBS strain COH31 was added to the macrophages as described for the adherence assay in DPBS containing both Ca^{2+} and Mg^{2+}, DPBS containing either Ca^{2+} or Mg^{2+} and DPBS without divalent cations. Macrophage monolayers were exposed to these same conditions without GBS to observe morphological changes in macrophage adherence to the chamber-slide during the incubation period with bacteria.

Treatment of macrophages with phorbol ester and/or ligation of the macrophage fibronectin receptor on the adherence of GBS by macrophage monolayers Preparations of macrophage monolayers was performed as described previously using eight-chamber tissue culture slides (Lab-Tek) having a plastic surface which enhances protein coating. Prior to addition of the peritoneal exudate cells, the plastic slide surfaces were coated with mouse serum albumin (1 mg/ml) or fibronectin (FN) from rat plasma (0.1 mg/ml) by a 60 min incubation at 20°C (115). The surfaces were washed three times with DPBS, incubated for 3 h at 37°C with the cells (0.25 ml/chamber). Where indicated phorbol dibutyrate (PDB) at 500 ng/ml was included during the plating of the cells. GBS strain COH31 was added to the macrophages as described for the adherence assay in the presence or absence of PDB (500 ng/ml). Macrophage monolayers were exposed to these same sets of condi-

tions without GBS to observe morphological changes in macrophage adherence to the chamber-slide.

Results

Trypsin sensitivity of the macrophage capacity to bind Group B streptococci

The effect of trypsin on the capacity of macrophage monolayers to bind GBS was studied after treatment of the cells with increasing doses of trypsin. Macrophage monolayers were treated with 4.0, 40, 400 and 4000 μg/ml of purified trypsin, washed twice with RPMI, incubated with aprotinin at concentration equimolar to the trypsin to inactivate the enzyme, washed again, and incubated under the usual conditions with 10^9 and 10^8/ml GBS. The number of microscopic fields analyzed in order to determine the percentage of 1000 macrophages binding GBS was the same for the trypsin/aprotinin-treated and aprotinin-treated (control) monolayers. The binding of five or more bacteria, which was exhibited by about 55% of the untreated macrophages, for each isogenic strain (Fig. 1), was relatively little affected by exposure to 4.0 μg/ml of trypsin. GBS binding fell to less than 40% after treatment with 40 μg/ml for each strain. Increasing the dose of trypsin further diminished the capacity of the macrophages to bind bacteria; and at trypsin concentratons of 4 mg/ml, less than 10% of the macrophages were able to bind GBS. Binding of GBS, without terminal sialic acid or without

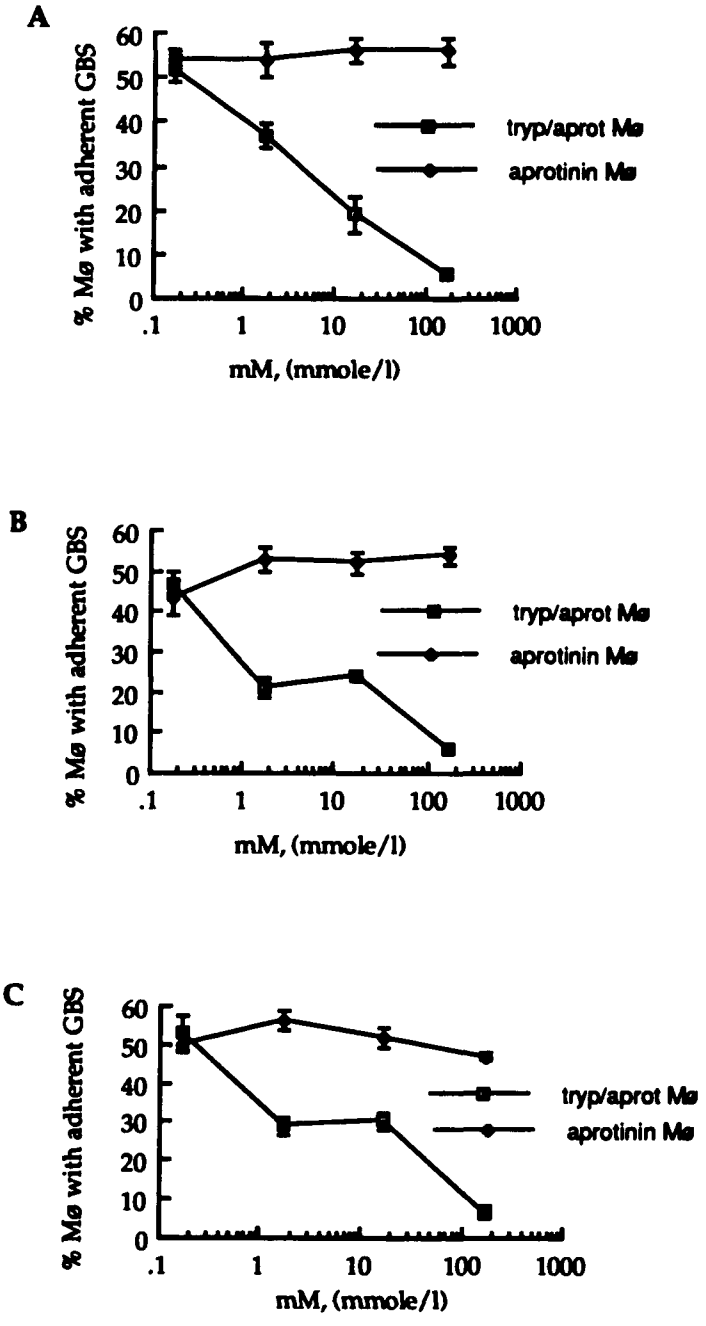

Fig. 1. Effect of trypsin treatment of Mø on GBS binding. (A) strain COH31, (B) strain COH31/15, (C) strain COH31/21

capsule, may be significantly less than the binding of wild-type COH31 by monolayers treated with 40 μg/ml although there are no apparent differences when monolayers are treated with higher doses of trypsin.

Effect of neutrophil elastase on macrophage binding to GBS Macrophage monolayers were treated with increasing concentrations of neutrophil elastase over the range of 3.0 to 30 μg/ml and washed exhaustively with DPBS to remove the enzyme prior to the addition of wild-type COH31 GBS. Treatment of macrophages at each concentration of elastase had no effect on the percentage of macrophages binding five or more bacteria, determined by counting 1000 macrophages, when compared to bacterial binding by untreated macrophages. The percentage of macrophages binding five or more bacteria remained about 55%. In addition, there was no morphological change, at 1000 X magnification, of the elastase-treated macrophage monolayers.

Effect of 2-deoxy-D-glucose treatment of macrophages on the binding of GBS Macrophage monolayers were treated with 2-deoxy-D-glucose over the range of 0.5 to 500 mM for 2 h prior to the addition of bacteria as well as during the period of bacterial binding. Fig. 2 shows that approximately 55% of untreated macrophages bound five or more wild-type COH31 GBS, determined by counting 1000 macrophages. At the lowest concentration of 2-deoxy-D-glucose, 0.5 mM, there was a decrease in binding to 38% macrophages, followed by an increasing reduction in binding of GBS by macrophages at the

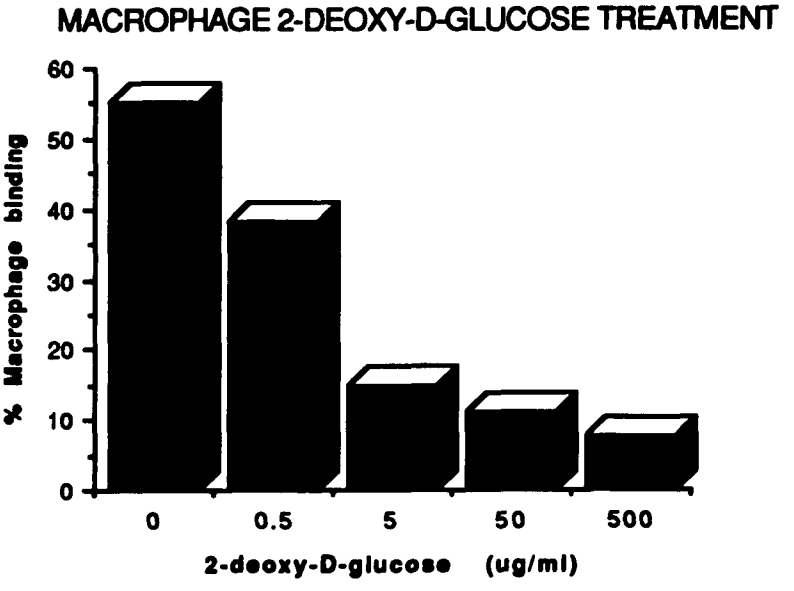

Fig. 2. Effect of pretreatment of Mø with the phagocytosis inhibitor, 2-deoxy-D-glucose, on adherence of GBS to these cells.

higher concentrations of 2-deoxy-D-glucose. A decrease in binding was maximal at 50 mM, resulting in less than 10% of the macrophages binding bacteria.

Temperature and divalent cation dependence of GBS binding A study of the effect of temperature on the binding of GBS by macrophage monolayers revealed dramatic differences in the percentage of macrophages binding five or more bacteria. As shown in Fig. 3 binding was about 60% at 37°C. Binding was lowered three-fold to 22% at 20°C and was almost absent (3%) at 4°C.

There is an absolute requirement for either Ca^{2+} or Mg^{2+} to support adherence of GBS to the macrophage monolayers as shown in Fig. 4. However, the presence of both cations, at 0.25 mM each, increased the percentage of macrophages binding bacteria from about 35% for either cation to 60%. This is the maximum binding I have ever achieved in any of my studies where DPBS was used during the incubation of GBS with the macrophage monolayers. DPBS (Sigma Chemical Co,) contains 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+}.

Effect of phorbol ester and/or ligation of fibronectin receptors on GBS binding In this study I was investigating the effect of phorbol dibutyrate (PDB) on the ability of macrophages to bind GBS. In addition, I studied the effect of ligation of the macrophage fibronectin receptor, by itself or in combination with the presence of phorbol ester, on the binding of bacteria. Figure 5 depicts the effects of phorbol ester and ligation of the fibronectin receptor on the

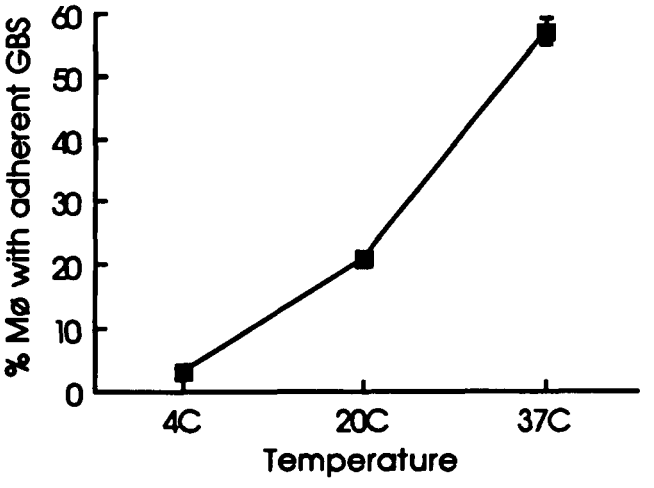

Fig. 3. Effect of temperature on the binding of GBS to murine peritoneal Mø

(N = 8)

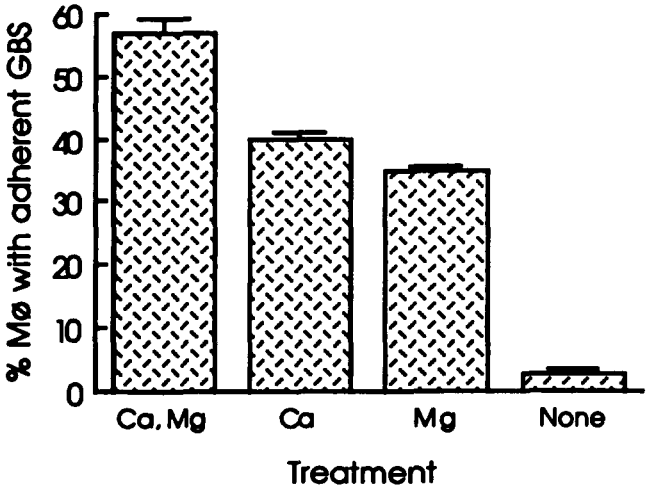

Fig. 4. Role of divalent cations on the adherence of GBS to murine peritoneal Mø

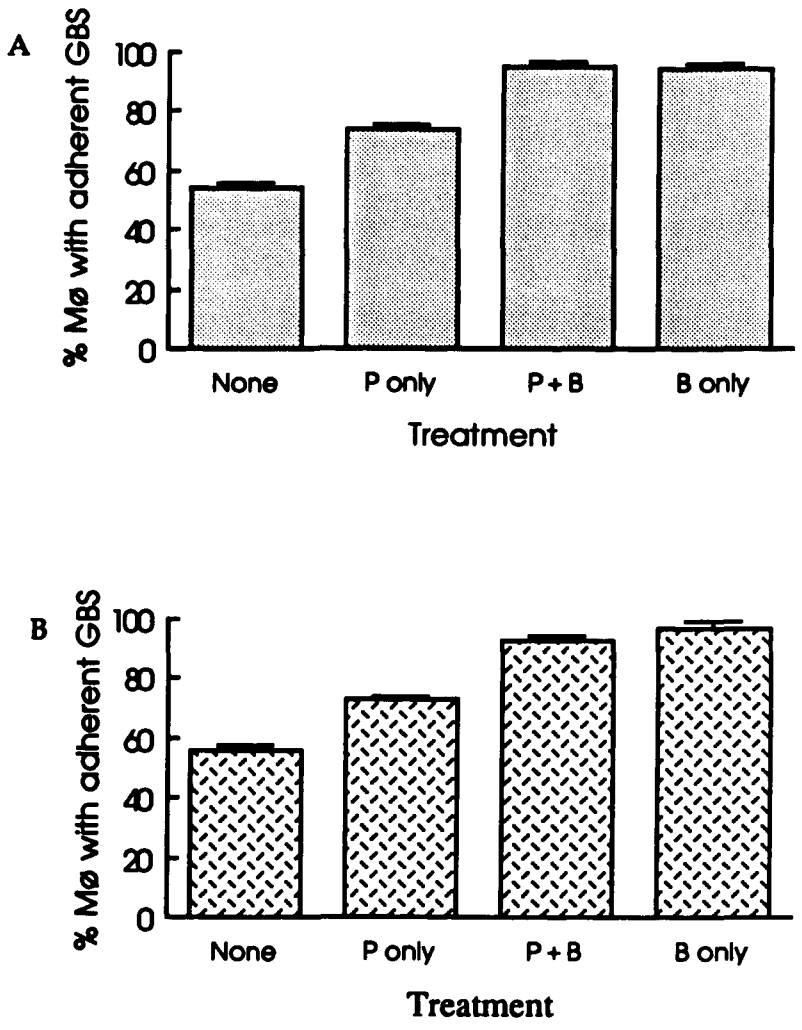

Fig. 5. Effect of phorbol ester and ligation of fibronectin receptor on binding of GBS to murine peritoneal Mø. (See following page.)

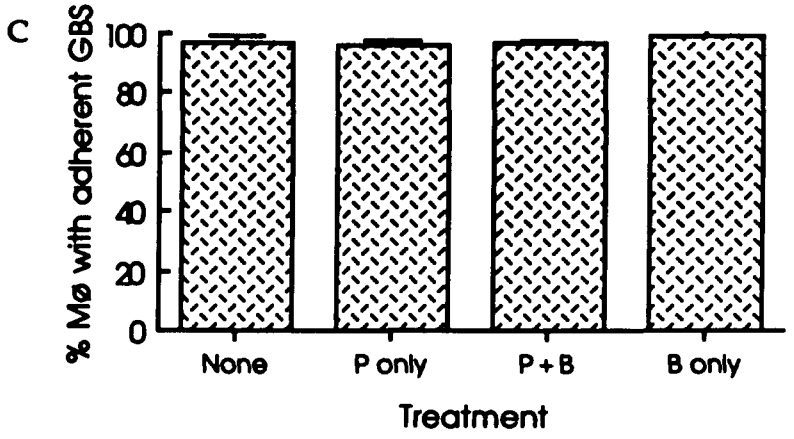

Fig. 5. Effect of phorbol ester and ligation of firbonectin receptor on binding of GBS to murine peritoneal Mø. (A) binding to uncoated surface, (B) binding to albumin-coated surface, (C) binding to fibronectin-coated surface. KEY: ***none*** = no phorbol ester present; ***P only*** = phorbol ester present only during the plating of Mø; unbound ester removed prior to addition of bacteria; ***P & B*** = phorbol ester present during both plating of Mø and incubation of Mø with bacteria; ***B only*** = phorbol ester present only during incubation of bacteria with Mø

percentage of macrophages binding five or more bacteria. When macrophages were allowed to adhere to an uncoated or albumin-coated surface, percent binding was similiar, about 55%. However, when PDB was present during the incubation of macrophages and bacteria with an uncoated or albumin-coated surface, the percentage binding almost doubled (94%). When PDB was present only during the plating of macrophages to the uncoated or albumin-coated surface of the slide and then washed away when non-adherent peritoneal exudate cells were removed, the percentage binding was lower (70%) than when PDB was present with bacteria, although this percentage binding was still higher than with no PDB treatment. When macrophages were plated on fibronectin-coated surface, with no PDB treatment, percentage binding as determined by the visual assay was maximal, 96%. The presence of PDB during plating only, during plating and binding or during binding only had no effect on the percentage macrophage binding GBS on a fibronectin coated surface. Thus both exposure to PDB during incubation of bacteria with Mø and ligation of the fibronectin receptor lead to an increase in bacterial binding to the Mø. If PDB is present during the plating of macrophages and then washed away, the effect of increased binding diminishes.

Morphological changes were observed for those macrophages plated on a fibronectin coated surface as compared with those attached to the albumin-coated or uncoated surfaces. The cells appeared to be covering a greater

surface area per cell and to be more stellate in shape. The cytoplasm to nucleus ratio was increased, so that attached or internalized bacteria were much more visible.

Discussion

A number of studies have been described in this paper on the effect of various enzymes, chemical treatments and temperature on the binding of GBS to monolayers of murine peritoneal macrophages in the absence of serum. The purpose of these experiments was to elucidate a mechanism of non-opsonin-mediated recognition of bacteria by phagocytic cells in order to have a better understanding of our innate host defenses. Innate immunity is particularly important for individuals in which the immune system may not be operating optimally, for example in geriatric individuals, infants, individuals with a compromised lymphocyte immunity, and for certain sites in the body such as the lungs in which low serum opsonin concentrations may not be sufficient to effectively opsonize many bacterial species (28). Previous studies in our laboratory have demonstrated that this binding is not mediated by the interaction between galactose exposed on GBS and the galactose receptor on the macrophage (see Chapter 2).

The trypsin sensitivity of the macrophage mechanism involved in the recognition of GBS is compatible with the function of a membrane protein.

This recognition mechanism appears to be distinct from known macrophage receptors such as the Fc receptor and the C3b receptor, CR1, whose activities are resistant to trypsin digestion (36). In addition, in Fig. 1 I observed that the presence of capsule or exposure of terminal galctose on the GBS had no effect on macrophage binding. Working on the assumption that bacterial binding was mediated by a Mø membrane protein, I attempted to identify this receptor by comparing parameters of this binding with those of known macrophage receptors.

Neutrophil elastase has been implicated as a factor that impairs local host defenses in chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis. Tosi *et al.* (100) showed that this enzyme cleaves CR1 from neutrophils (PMN) in the lungs of infected patients. The C3bi receptor on these cells, CR3, is resistant to elastase. My studies with treatment of macrophage monolayers by increasing concentrations of elastase demonstrated no reduction in the binding of GBS at levels as high as 30 μg/ml. CR1 is sensitive to elastase at 10 μg/ml (100). Neutrophil elastase has been shown to cleave other phagocytic receptors including the Fc receptor (99).

Two-deoxy-D-glucose has been shown to inhibit Fc and CR3-mediated phagocytosis of mouse peritoneal macrophages but not mannose receptor-mediated phagocytosis (97). Phagocytosis does not require protein synthesis and protein glycosylation, processes interrupted by accumulation of metabolites of 2-deoxy-D-glucose in the cell. The mechanism of 2-deoxy-D-glucose

inhibition of phagocytosis is distinct and may involve a complex series of events resulting from a depolarized macrophage, a lowered cellular energy reserve, and may also involve the imbalance in the intracellular pool of ions critical for phagocytosis (97). Of interest is the finding that despite the large area of plasma membrane internalized by the macrophage during phagocytosis, concomitant protein synthesis is not required. Part of this may be due to the ability of macrophages to recycle their membrane proteins (54). There are also large numbers of membrane vesicles in the macrophage to replenish the internalized membrane (87).

The method used in this study for visual quantitation of GBS recognition by macrophage monolayers cannot differentiate between attached and internalized bacteria. Numbers of internalized bacteria are therefore included in the data as bound bacteria. Since binding is allowed to occur at 37°C and the macrophages are viable, macrophages with phagocytosed bacteria probably contribute to the number of cells scored as positive for binding five or more bacteria. Therefore, the property of dose-dependent inhibition of binding of GBS observed in our studies for 2-deoxy-D-glucose may involve CR3, which has been implicated in the direct binding of other pathogens (115).

The characteristics of temperature and divalent cations on the binding of GBS to macrophage monolayers were studied. Binding required either Ca^{2+} or Mg^{2+} and increased by 50% if both cations were present. This was not the result of a higher total concentration of ions; the total concentration of cation

present either individually or jointly was 0.5 mM. Binding was also temperature dependent with maximum binding occuring at 37°C. The requirements for divalent cations and warm temperatures are unlike those for the binding of lectin receptor (I. Ofek, personal commun.) and IgG or C3b-coated particles (116). However, these characteristics closely resemble the requirements for binding of particles by CR3 (112).

Receptors for the third component of complement (C3) on cultured human monocytes bind ligand-coated particles but do not initiate phagocytosis (32). The function of these receptors, however, is altered dramatically after monocytes attach to surfaces coated with fibronectin or after monocytes are exposed to phorbol esters. Fn and phorbol esters "activate" C3 receptors such that they promote vigorous phagocytosis (116). There is no evidence that Fc or lectin-like receptors are affected by these treatments. In my adherence assay, bacteria that have been internalized by phagocytosis probably contribute to the number of macrophages observed to bind five or more bacteria, therefore I studied the effect of macrophages treated with phorbol esters or plated on a fibronectin-coated surface on the binding of GBS.

In summary I have shown that ligation of FN receptors causes enhanced "binding" of GBS to macrophages. The effect of ligated FN receptors on the increase numbers of GBS associated with the macrophages can be mimicked by PDB. The effect of PDB on increased binding of GBS by macrophages was shown to be reversible when the phorbol ester was removed. PDB was used

in these studies instead of the more hydrophobic phorbol myristate acetate (PMA) since more than one-half of cell-associated PDB may be released from the cells upon washing 20°C (29).

This study confirms that a macrophage membrane receptor is involved in the binding of GBS *in vitro*. Table 1 summarizes data from my studies and others in comparing the effects of temperature, elastase, 2-deoxy-D-glucose, phorbol ester, ligation of FN receptors and divalent cation requirements with known macrophage receptors. A comparison of binding characteristics for the macrophage Fc receptor, galactose-receptor and integrin receptor for C3bi, suggest a similiarity between CR3 and the receptor for GBS.

TABLE 1

PROPERTIES OF KNOWN BACTERIAL RECEPTORS ON Mø

Properties	Receptors			
	GBS[a]	Integrin	Lectin	Fc
Temperature dependence	+	+ (116)[b]	– (105)	– (116)
Trypsin sensitivity	+	+ (16)	– (105)	– (16)
Elastase resistance	+	+ (100)	?	– (99)
Ca^{2+} *and* Mg^{2+} requirement	–	– (116)	+ (105)	+ (116)
Ca^{2+} *or* Mg^{2+} requirement	+	+ (116)	– (105)	– (116)
2-deoxy-D-glucose inhibition	+	+ (97)	– (97)	+ (97)
FN-substrate enhancement	+	+ (114)	– (114)	– (114)
Phorbol ester enhancement	+	+ (114)	– (114)	– (114)

[a] data from this study

[b] results compiled from references cited

CHAPTER 4

ROLE OF β_2 INTEGRINS AND LIPOTEICHOIC ACID IN THE ADHERENCE OF GROUP B STREPTOCOCCI TO MURINE PERITONEAL MACROPHAGES

Abstract

The type III group B streptococcus (GBS), an extracellular pathogen, is recognized by murine peritoneal macrophages (Mø) in the absence of serum components such as complement or antibody. To understand this non-opsonin mediated adherence of GBS, I have investigated the role of the Mø β_2 family of integrin receptors, CR3, LFA-1 and p150,95, and the bacterium-bound ligand, lipoteichoic acid, in the mechanism of adherence. Two monoclonal antibodies that bind to either subunit of murine CR3, M1/70 and M18/2, inhibited adherence of GBS to Mø; attachment was inhibited by 31 and 66% by M1/70 and M18/2, respectively. The interaction between GBS and CR3 probably does not involve the C3bi-binding site on CR3, however, because a monoclonal antibody which exhibits specificity for this site, M1/70, inhibited GBS attachment by only 31%. In contrast, M18/2, which reacts with the β subunit common to all members of this integrin family, inhibited ingestion by 66%. The effect of using both monoclonal antibodies was not

additive. Downmodulation studies using the same antibodies, separately and in combination, to deplete the receptors from the apical surface of adherent Mø were performed to examine their effect on GBS adherence. Similiar results were obtained. Results from flow cytometry studies using these same antibodies in solution with a mixture of suspended bacteria and Mø also showed similiar inhibition profiles. One monoclonal antibody, 5C6, that ligates a different epitope on the α-subunit of CR3 from M1/70, did not produce an inhibitory effect on the adherence of GBS to Mø by flow cytometry. I was unable to use this antibody in downmodulation studies or inhibition studies requiring a Mø monolayer because this antibody recognizes a Mg^{2+} -dependent anchorage site on the Mø.

A dose-dependent effect of LTA to inhibit the binding of GBS to Mø was observed in all studies using LTA; downmodulation of Mø receptors by LTA, LTA in solution with Mø monolayers or in flow cytometry studies with suspended cells. Inhibition of GBS adherence to Mø was maximal (70%) at a concentration of 30 μg/ml.

My studies suggest the role of LTA and the β_2 integrins in the binding of GBS by murine peritoneal Mø. Recognition of phosphosugar surface structures on the extracellular pathogen, GBS, is consistent with other studies suggesting that a similar surface struture on both intracellular and extracellular bacteria is recognized by the CR3, LFA-1 and p150,95 family of leukocyte integrins.

Introduction

Adhesion between phagocytes and other cells, particles, or the extracellular matrix is crucial for a variety of functions such as phagocytosis, diapedesis, and positioning of phagocytes in the liver, lungs, and other tissues (103). Phagocytosis represents an important effector mechanism for the eradication of infectious agents. It is performed primarily by specialized cells of two different lineages, namely polymorphonuclear neutrophilic granulocytes (PMN) and mononuclear phagocytes (Mø). Because of their major function, these cells are often referred to as 'professional phagocytes' to distinguish them from most other host cells which are called 'non-professional phagocytes'. To fulfill these functions, PMN and Mø must respond to specific signals that indicate the presence of infectious agents and also collaborate with the elements of specific immunity. Phagocytosis alone cannot eradicate invading microbes, it must be followed by intracellular killing mechanisms using specialized organelles and molecules. It has long been assumed that PMN and Mø have specific receptors for binding microorganisms directly and specific receptors that mediate cellular adhesions, but only recently have such receptors been isolated and characterized.

The idea that a few receptors could be responsible for a wide range of adhesion events first arose with the discovery of a class of patients that exhibit recurrent life-threatening infections with gram-positive, gram-negative and

fungal pathogens (5). These patients exhibit extreme leucocytosis but fail to form pus at sites of infection. *In vitro* experiments showed that PMN from these patients are defective in their ability to adhere to C3bi-coated erythrocytes, to protein-coated glass or plastic surfaces (3), and to endothelial cells (34), and that failure to adhere results in the inability to display chemotactic responses. Thus, susceptibility to infection probably results both from an inability to bind and ingest opsonized pathogens and a failure to recruit cells to sites of infection. Additionally, these observations suggest that the leucocytes of patients fail to extravasate and are retained in the vasculature by a failure to adhere to endothelial cells, giving the name of leucocyte adhesion deficiency (LAD) to this condition. Characterization of the proteins missing from the patients' leucocytes have confirmed their role in adhesion to endothelium and in several other adhesion events.

LAD is caused by a failure of leucocytes to express three related proteins, LFA-1, CR3 and p150,95, of the integrin family of cell-cell and cell-matrix proteins (90). Each of these cell-surface glycoproteins consists of an $\alpha_1\beta_1$ dimer composed of a 150,-190,000 M_r α chain and a 95,000 M_r β chain, schematically depicted in Fig. 1. The β chain is identical in each of these three proteins (77) and has been given the designation CD18 by the International Workshop on Leucocyte Antigen Differentiation. The α chains are structurally and antigenically distinct (113). The α chains of LFA-1, CR3 and

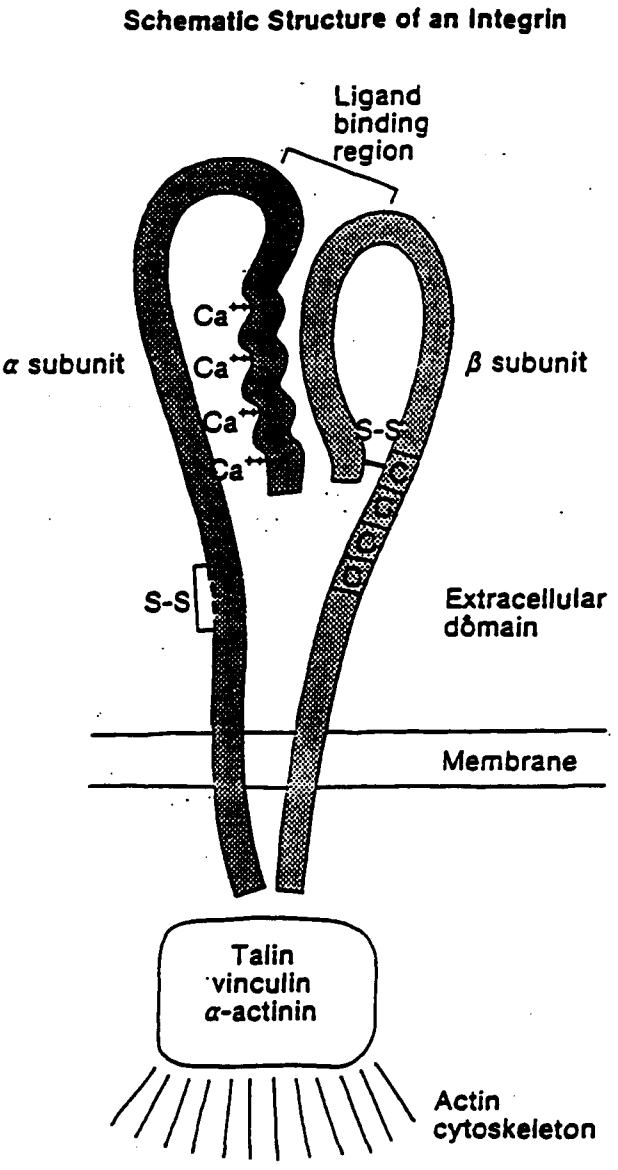

Fig. 1. Schematic structure of an integrin. Taken from Steven M. Albelda and Clayton A. Buck. 1990. Integrins and other cell adhesion molecules. FASEB J. 4:2868-2880.

p150,95, are termed CD11a, CD11b and CD11c, respectively, and CR3 is referred to as CD11b/CD18.

LAD patients are deficient in all three of these proteins because of an inherited defect in the β chain (90). Cells from LAD patients do synthesize normal precursors of the α chains, but these precursors are very rapidly degraded and do not appear on the cell surface. Patients have recurring infections, often fatal in childhood unless they are corrected by bone marrow transplantation. In a study by Ross *et al.* (73) , children with LAD had recurrent skin infections with *Staphylococcus epidermidis* but not yeast suggesting the role of CR3 and LFA-1 in the phagocytosis of unopsonized bacteria.

Lymphocytes express abundant LFA-1, but neither CR3 or p150,95. PMN express abundant CR3, whereas LFA-1 and p150,95 are minor but easily detectable components. Macrophages express large amounts of all three of these proteins. Expression of these proteins appears to be restricted to leucocytes; they have not been found on other mammalian cell types.

The first molecule shown to be deficient from LAD patients (5) had been previously identified as a receptor for a cell-bound fragment of complement (114) and was named CR3 (complement receptor type three). CR3 has also been referred to as Mac-1 and Mo1 because it was the first phagocyte-specific antigen identified by monoclonal antibodies in mouse and man, respectively. CR3 functions as an opsonic receptor, and promotes the binding of C3bi-

coated cells and particles by monocytes, macrophages and PMN (112, 117). In appropriately stimulated cells, binding is followed by phagocytosis of the particle but does not induce reactive oxygen metabolites (21). Therefore, CR3 provides a safe way for entry into professional phagocytes, compared to internalization via the Fc receptor. Most intracellular pathogens except viruses, have been shown to use CR3 for entry into mononuclear phagocytes. Examples are *Legionella pneumophila,* (63) Mycobacterium tuberculosis, (64), *Leishmania* sp. (98), and *Histoplasma capsulatum* (12).

CR3 requires relatively high concentrations of divalent cations (about 0.5 mM Ca^{2+} and Mg^{2+}) in order to interact effectively with ligand (112). Integrin α-subunits have three or four tandem repeats of a putative divalent cation-binding motif, and have been shown to require Ca^{2+} or Mg^{2+} for function (91). This behavior contrasts with that of other opsonic receptors (CR1 and FcR) which do not require divalent cations for binding activity. In addition, the binding capacity of CR3 is temperature dependent, and is absent in cells held at 0°C (117), again distinguishing CR3 from other opsonic receptors (CR1 and FcR) which bind well at 0°C.

In the presence of serum, most pathogens are taken up via C3bi deposition on their surface. CR3 does not recognize either the precursor C3b, or the product of further cleavage C3dg (14, 72 ,112). Binding of monomeric or dimeric C3bi to CR3 has not been reported, presumably because of low binding affinity. Studies using sheep erythrocytes coated with 10^4 to 10^5 C3bi per

cell demonstrate avid binding to CR3-bearing cells presumably because of multivalent interaction with the phagocytes. By analogy, it is likely that LFA-1 and p150,95 also bind their targets with low affinity and that adhesion events mediated by these receptors are driven by multivalent interactions. A functional consequence of low affinity binding is the potential to reverse adhesion and promote detachment.

CR3 has two distinct binding sites. One is located on the α chain and binds ligands such as C3bi, containing the Arg-Gly-Asp (RGD) sequence. In the absence of C3bi, microorganisms expressing a surface protein containing the RGD sequence could bind to CR3. For example, *Bordetella pertussis* , a gram-negative coccobacillus and the causative agent of whooping cough, has been shown to adhere to human macrophages, *in vitro*. The interaction between macrophage CR3 and *B. pertussis* filamentous hemagglutinin (FHA) involves recognition of the RGD sequence at positions 1097-1099 in FHA (68).

The other binding site on the α chain of CR3 has a lectin-like character and, for example, recognizes lipopolysaccharide (LPS) located on the outer leaflet of the outer membrane of *Escherichia coli* (116). *Histoplasma capsulatum* binds directly to CR3 via the lectin-like binding site (12). In addition, an abundant surface glycolipid, promastigote lipophosphoglycan (LPG), from *Leishmania* has been shown to bind to the 'lectin-like-LPS' binding site on the α chain of CR3 (76, 98). It is possible that LPS, LPG and *H. capsulatum* display a common structure, such as a sugar phosphate, that can

be recognized by CR3 (116).

Characterization of the non-opsonic binding parameters of Group B streptococci (GBS) and murine peritoneal macrophages in our laboratory (see Chapter 3) has suggested a similiar binding profile for the 'GBS-receptor' and CR3, based on divalent cation and temperature dependence, trypsin sensitivity, elastase resistance and 2-deoxy-D-glucose inhibition. Group B streptococci contain membrane teichoic acid, lipoteichoic acid (LTA) which is known to mediate the adherence of GBS to human embryonic, fetal and adult epithelial cells (55). Hydrophobic interactions due to the lipid fatty acid portion of the polymer and possibly other cell surface components within the plasma membrane of embryonic and fetal cells has been suggested as a mechanism for adherence. Interaction of the glycerol phosphate backbone with specific receptor sites on the cell may follow or occur independently for the attachment of GBS to adult cells (55). The teichoic acid moiety of GBS LTA (55) and Group A streptococci LTA (58) has been implicated in the ability of these microorganisms to adhere to neonatal tissue. Competition binding experiments using soluble LTA may help to confirm if a structure such as a sugar phosphate, common to LTA, LPS, and LPG in involved in the binding of GBS to murine peritoneal macrophages. Positive evidence would support the theory that the GBS receptor is CR3.

Materials and Methods

Chemicals All chemicals were obtained from Sigma Chemical Co, (St. Louis, MO) unless otherwise noted.

Bacteria Type III Group B streptococci, designated as strain COH31, was kindly provided by Dennis Kasper, Channing Laboratory for Infectious Disease (Harvard Medical School). This strain was originally isolated from infants with meningitis (44). It was grown and maintained in our laboratory as previously described (see Chapter 2).

Assay of GBS adherence Monolayers of macrophages (prepared as previously described in Chapter 2) were layered with 0.2 ml of GBS at densities of 10^{10}/ml and 10^{9}/ml in DPBS and incubated at 37°C in the CO_2 chamber for 1 h as previously described (see Chapter 1).

Hybridoma lines and MAb The characteristics of the antibodies used in this study are summarized in Table 1. The derivation of rat anti-mouse subcloned lines of M1/70.15.11.5 (89), M18/2a.12 (78) and 5C6 (71) has been previously described. The sub-clones M1/70.15.11.5 (ATCC TIB 128) and M18/2a.12.7

TABLE 1

RAT ANTI-MOUSE MAb TO β_2 INTEGRINS

MAb	Origin	Epitope	Isotype
M1/70.15.11.5[a]	T. A. Springer (89)	α subunit of CR3, inhibits C3bi binding	IgG2b
5C6	S. Gordon (71)	α subunit of CR3, inhibits Mg^{2+}-dependent binding	IgG2b
M18/2a.12.7[a]	T. A. Springer (78)	β_2 subunit of CR3, LFA-1, and p150,95	IgG2a

[a] purified from hybridoma supernatants by protein G affinity chromatography.

(ATCC TIB 218) were obtained as frozen suspensions (American Type Culture Collection, Rockville MD). For brevity, these subclones will be designated as M1/70 and M18/2. The 5C6 antibody was purchased as purified IgG from Serotec (Kidlington, Oxford, designated as MCA 711).

To obtain large quantities of purified MAb, the hybridoma line of M1/70 was grown in Dulbecco's modified Eagle's medium (D-MEM) with 4.5 g/L glucose and the M18/2 line was grown in RPMI 1640, with 5×10^{-5} M 2-mercaptoethanol. Both media were supplemented with 10% low IgG fetal bovine serum (Hyclone Laboratories, Logan, Utah), 2mM L-glutamine and penicillin-streptomycin. The cell lines were grown in 75 cm^2 flasks, at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, to densities exceeding 10^6/ml to exhaust the media. The supernatant, containing secreted antibody, was removed from the cells by centrifugation (100 x*g*). MAb were purified by 50% saturated $(NH_4)_2SO_4$ precipitation and affinity chromatography on Protein G Superose® 4 Fast Flow (Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with 20mM phosphate-buffered saline, pH 7.0 (PBS), containing 0.1% thimerosal. The IgG was eluted with 0.1 M glycine, pH 2.7, and collected as 1.5 ml fractions in tubes containing 45µl 1.0M Tris-HCl, pH 9.0 to preserve the activity of the acid labile IgG. Column size and flow rates were determined by the rat IgG_{2a} and IgG_{2b} binding properties quoted in the data sheet accompanying the affinity chromatography product.

Lipoteichoic acids Purified and lyophilized lipoteichoic acid from *Streptococcus pyogenes* (group A streptococci) prepared in the laboratory of the late Edwin H. Beachey, University of Tennessee (Memphis, TN) was kindly provided by Itzak Ofek, Tel Aviv University (Tel Aviv, Israel). Purified LTA from *Streptococcus agalatiae* GBS was kindly provided by Stephen J. Mattingly, University of Texas, (San Antonio, TX). Solutions of each type of LTA at 10 mg/ml were prepared in sterile water to prevent deacylation. The LTA was diluted into DPBS, pH 7.2, immediately before use to the concentrations desired for the inhibition assays.

Inhibition studies using MAbs or LTA Macrophage monolayers were preincubated for 30 min at 37°C with soluble MAbs or LTA in DPBS before the addition of bacteria to study their effect on GBS adherence to peritoneal macrophages. Solutions containing various MAbs, alone or in combination, each at a final concentration of 10 and 50 μg/ml, or LTA at 75, 150, 300 μg/ml were added to the monolayers. Normal rat IgG and FBS were used as negative controls for the antibody studies. The effect of group A streptococcal LTA on binding was also studied by co-incubation of the monolayers with bacteria and the LTA, without the 'head start'.

Downmodulation Studies The ability of solid phase MAb to down-modulate macrophage CR3 and inhibit GBS binding was examined by coating 8-chamber glass slides (Lab-Tek) with the MAbs, alone or in combination, at

concentrations of 10 and 50 μg/ml in 0.1 M carbonate-bicarbonate, pH 9.6 overnight at 4°C. The effect of macrophages adhering to a surface coated with group B streptococcal LTA on bacterial binding was also studied. Since LTA contains both hydrophobic and hydrophilic regions, I allowed LTA to adhere to both plastic and glass slides for my studies of inhibition of adherence. It was possible that one of these surfaces would provide a better substrate for LTA, based on its different regions. The slides were coated overnight at 4°C with DPBS containing LTA at 30, 300 and 3000 μg/ml. After removing unbound MAbs or LTA with DPBS, peritoneal exudate cells were added to these slides as described above to prepare macrophage monolayers. Bacteria were added to these slide for the binding assay. Rat IgG and FBS were used to coat slides as negative controls for inhibition.

Flow cytometry To examine the adherence of GBS by macrophages in suspension, flow cytometric analysis of fluorescent GBS bound to macrophages was performed by the use of a FACStar^{PLUS} (Becton Dickinson, FACS Systems, Sunnyvale, CA) with computer-assisted evaluation of data (FACStar^{PLUS} Lysys II software). To label the bacteria with a fluorochrome, I chose Lucifer Yellow VS. Lucifer Yellow (LY) (95) is the vinyl sulfone derivative of a sulfonated 4-amino napthalimide molecule with spectral properties similiar to the more commonly used fluorescein isothiocyanate. Unlike the fluorescein, however, the fluorescence intensity of LY is stable from pH 2 to pH 10.

Thus, LY fluorescence is unaffected by pH changes achievable in endocytic vesicles or lysosomes (48). When LY was linked covalently to amino and sulfhydryl groups on the surface of *Streptococcus pneumoniae*, Sveum *et al.* (95) reported quantitation of macrophage-associated bacteria at all stages of the adherence and ingestion phases of phagocytosis.

Labelling of bacteria was achieved by incubating 10^{10} GBS and 1 mg of Lucifer Yellow VS (LY) in 1.0 ml of 0.1 M $NaHCO_3$, pH 9.5, for 2 h at room temperature, then washing the cells five times in DPBS (95). Washed peritoneal exudate cells containing 95% macrophages at 2 x 10^6/ml and LY-GBS at 10^9/ml, in DPBS, were incubated for 1 h at 37°C to allow for binding to occur. Cells were fixed by addition of an equal volume of 2% formaldehyde and samples were either examined immediately or stored at 4°C for up to two weeks before analysis by flow cytometry.

The effect of MAb or GBS-LTA on adherence of GBS was investigated by preincubating the macrophages for 30 min at 37°C with MAb M1/70, M18/2 or 5C6, at 10 and 100 μg/ml, or LTA at 30 and 300 μg/ml, before the addition of bacteria. The emission of Lucifer Yellow-labeled GBS attached to macrophages was recorded at 530 nm after excitation by the argon laser at 488 nm. Data were acquired by using an instrument status with a linear data mode for Forward Scatter (FSC) and Side Scatter (SSC) and a logarithmic data mode for Filter 1 (Fl1) for 530 nm. The FSC threshold was raised to eliminate unbound bacteria, red blood cells, platelets and and a live gate was set around the macro-

phages in the control.

The Kolmogorov-Smirnov (K/S) Two-Sample Test was used to test for inhibition of bacterial adherence to macrophages by MAb or LTA. K/S calculates the probability that two histograms are different by computing the summation of the curves and finds the greatest difference between the summation curves.

Results

A macrophage β_2 integrin mediates adherence of group B streptococci To explore the role of the macrophage β_2 integrins in mediating adherence of GBS to peritoneal macrophages, I examined the capacity of MAb directed against either subunit to inhibit bacterial adherence. The cumulative data from two experiments, using soluble antibodies and macrophage monolayers, are shown in Table 2. MAb M18/2, directed against the β-subunit CD18 common to all three members of the β_2 integrins, significantly inhibited GBS adherence. Monoclonal antibody M1/70, directed against the C3bi binding site on the α-subunit, CD11b, also inhibited GBS adherence but to a lesser degree. The mean inhibition for M18/2 was 66% and mean inhibition for M1/70 was 31%. When the antibodies were used in combination the mean inhibition was 70%. When the monoclonal antibody 5C6, with specificity for the Mg^{2+} -dependent anchorage site on CD11b, was tested for its effect on

TABLE 2

INHIBITION OF STREPTOCOCCAL ADHERENCE TO Mø BY MONOCLONAL ANTIBODIES

Antibodies Used			Percent Inhibition[a]		p Value[b]
M1/70	M18/2	Polyclonal IgG			
–	–	–	0	0	—
–	–	+	3	3	—
–	+	–	64	67	0.006
+	–	–	33	28	0.008
+	+	–	67	72	0.001

[a] Results of two separate experiments

[b] Based on analysis of variance. p Values based on comparisons between effect of each MAb and IgG control. There was no significant difference between IgG control and control with no antibody added. There was no significant difference between the effect of M1/70 alone and in combination with M18/2 ($p = 0.304$).

inhibition of GBS adherence, the macrophages appeared to become less adherent and many were removed from the surface of the slide with the subsequent washings. Therefore, whenever this antibody was included in my tests I was unable to obtain results for its effect on macrophage binding of GBS. Control antibody, polyclonal rat IgG, did not significantly inhibit GBS adherence (mean inhibition, 3%).

Downmodulation studies using antibodies M1/70 and M18/2 demonstrated a similar inhibition profile. As shown in Table 3 the mean inhibition of GBS adherence for macrophages attached to a M18/2 -coated surface, from two experiments, was 59% and the mean inhibition for M1/70 was 28%. When both antibodies were used, mean inhibition was 62%. Macrophages attached to the glass slide coated with MAb 5C6 appeared to be very unstable. The cells were not well attached (*i.e.*, they appeared rounded up) and were easily removed by washing with DPBS. Therefore this antibody was not used in the downmodulation studies. When macrophages were allowed to attach to a polyclonal rat IgG-coated surface there was no significant inhibition of GBS binding.

When flow cytometry was employed to study the binding of GBS to macrophages it was possible to quantify bacterial binding without having to wash away unbound bacteria. Figure 2 compares the fluorescence intensity of unstained GBS with that of LY-labelled GBS. For this method we kept the macrophages in suspension by using siliconized microcentrifuge tubes;

TABLE 3

EFFECT OF DOWN-MODULATION OF INTEGRIN ON STREPTOCOCCAL BINDING TO Mø

Ab used	Specificity	Percent Inhibition[a]		p Value[b]
None	—	0	0	—
IgG	polyclonal	3	3	—
M1/70	CR3 α chain	26	30	0.006
M18/2	β_2 chain	57	61	0.001
M1/70 + M18/2	α and β_2 chains	60	64	0.001

[a] Results of two separate sets of experiments. Positive binding: ≥ 5 bacteria/MØ. 1000 MØ counted for each entry

[b] Based on analysis of variance. p Values based on comparisons between effect of each MAb and IgG control. There was no significant difference between IgG control and control with no antibody added. There was no significant difference between the effect of M1/70 alone and in combination with M18/2 ($p = 0.400$).

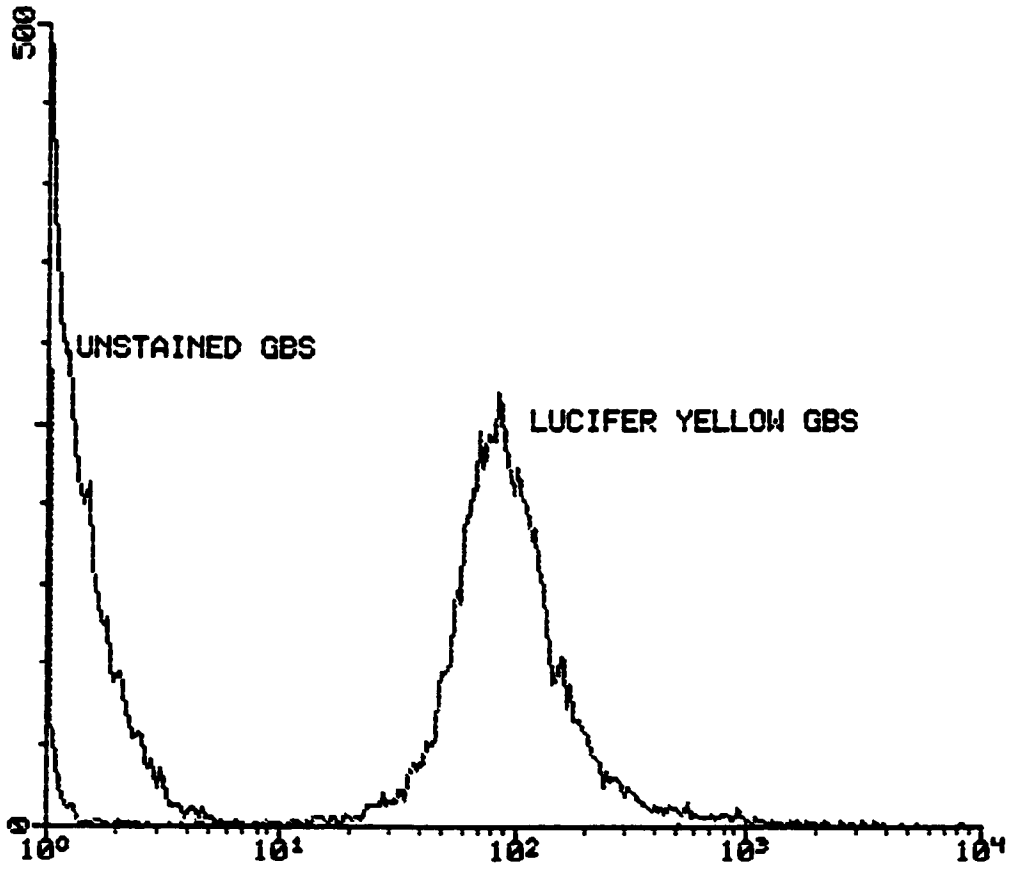

Fig. 2. Fluorescence histograms of unlabeled and LY-labeled group B streptococci.

therefore I was able to test the effect of MAb 5C6 (against the anchorage-dependent site) on the binding. Figures 3–5 are overlays of fluorescence histograms comparing the FSC with fluorescence intensity, for unstained GBS binding to macrophages, with maximum fluorescence of LY-labelled GBS binding to macrophages as well as the effect of MAb M18/2 , M1/70, and 5C6 on the fluorescence intensity of the GBS-bound macrophage.

The K/S statistic, D/s(n), is an index of similarity for two curves. [s(n) = square root of $(n_1 + n_2)/(n_1 * n_2)$, where n_1 is the number of events in the first histogram; n_2 is the number of events in the second histogram] If D/s(n) = 0, the curves are identical. A value of 5.0 or greater indicates a statistically significant difference between two curves. Table 4 summarizes the D/s(n) statistic for each concentration of MAb. Monoclonal antibody M1/70, recognizing CD11b (α_m-subunit) and M18/2, recognizing CD18 (β-subunit) each inhibited the adherence of GBS to macrophages. The monoclonal antibody 5C6, recognizing a different epitope on CD11b was not inhibitory. These data are consistent with downmodulation and soluble inhibition studies using MAbs, supporting the role of β_2 integrins in the binding of GBS.

The role of LTA in the recognition of GBS by peritoneal macrophages The effect of soluble LTA from group A and group B streptococci on GBS binding by macrophage monolayers was studied in two separate experiments. Figure 6 shows the cumulative data from both studies. For my first studies only

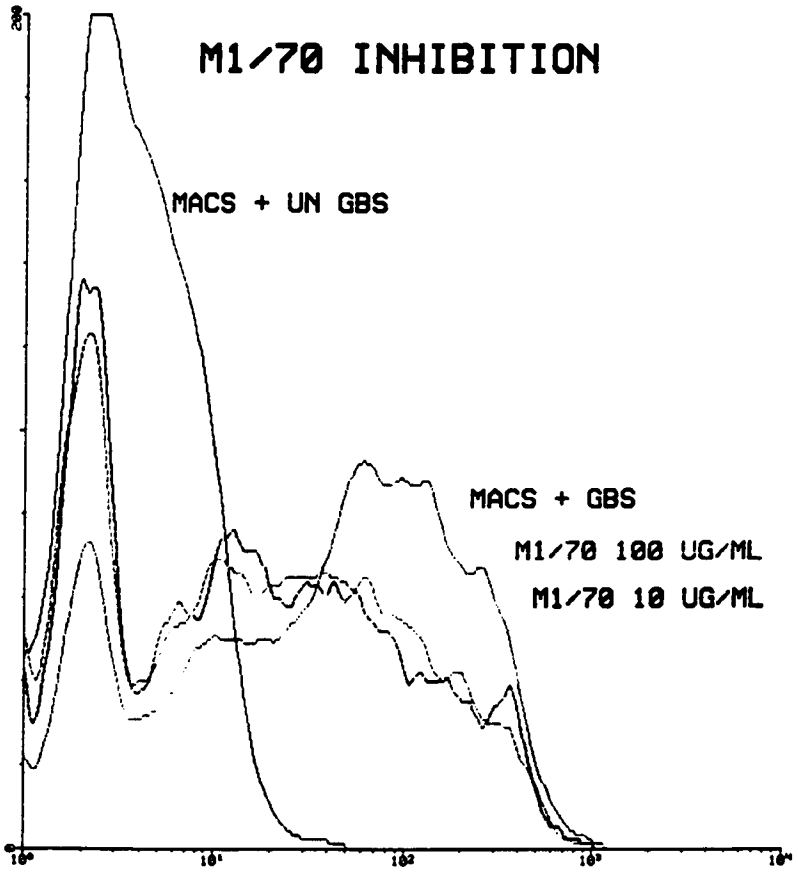

Fig. 3. Effect of monoclonal antibody M1/70 on adherence of LY-labeled GBS and Mø. Overlay of histograms for controls: Mø + unlabeled GBS demonstrates inherent fluoresecence of Mø; Mø + LY-labeled GBS demonstrates maximum fluorescence intensity without antibody.

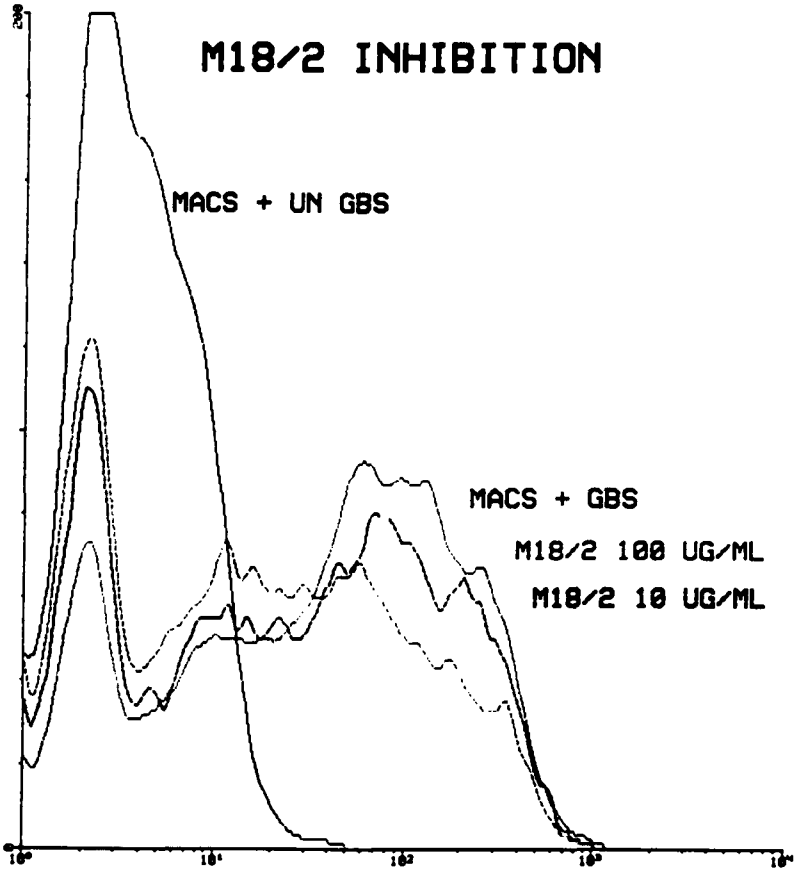

Fig. 4. Effect of monoclonal antibody M18/2 on adherence of LY-labeled GBSand Mø. Overlay of histograms for controls: Mø + unlabeled GBS demonstrates inherent fluoresecence of Mø; Mø + LY-labeled GBS demonstrates maximum fluorescence intensity without antibody.

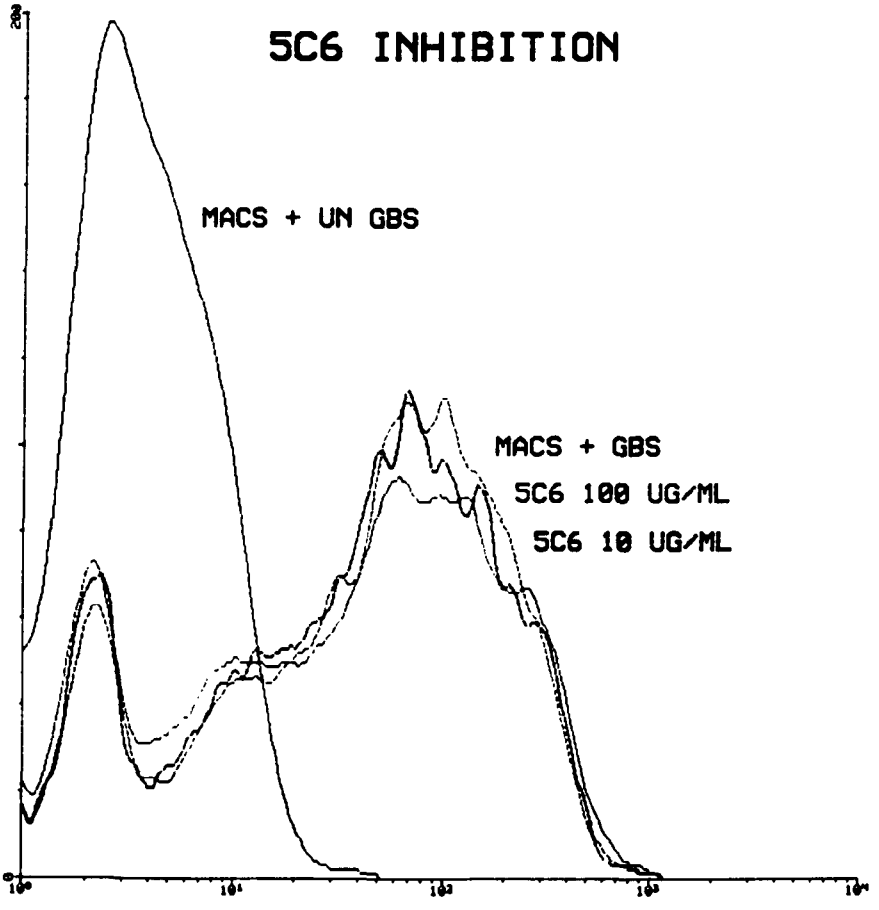

Fig. 5. Effect of monoclonal antibody 5C6 on adherence of LY-labeled GBS and Mø. Overlay of histograms for controls: Mø + unlabeled GBS demonstrates inherent fluoresecence of Mø; Mø + LY-labeled GBS demonstrates maximum fluorescence intensity without antibody.

TABLE 4

KOLMOGOROV-SMIRNOV ANALYSIS OF FLUORESCENCE HISTOGRAMS ASSESSING THE EFFECT OF MONOCLONAL ANTIBODIES TO LEUKOCYTE INTEGRINS ON BINDING OF GROUP B STREPTOCOCCI TO MURINE MACROPHAGES

Concentration	Monoclonal Antibody		
	M1/70	M18/2	M5C6
10 μg/ml	11.67*[a]	3.23	1.75
100 μg/ml	10.65*	10.05*	2.68

[a] Numbers refer to D/s(n) values. See text for details. Values greater than 5.0 are considered statistically significant and are noted by an asterisk.

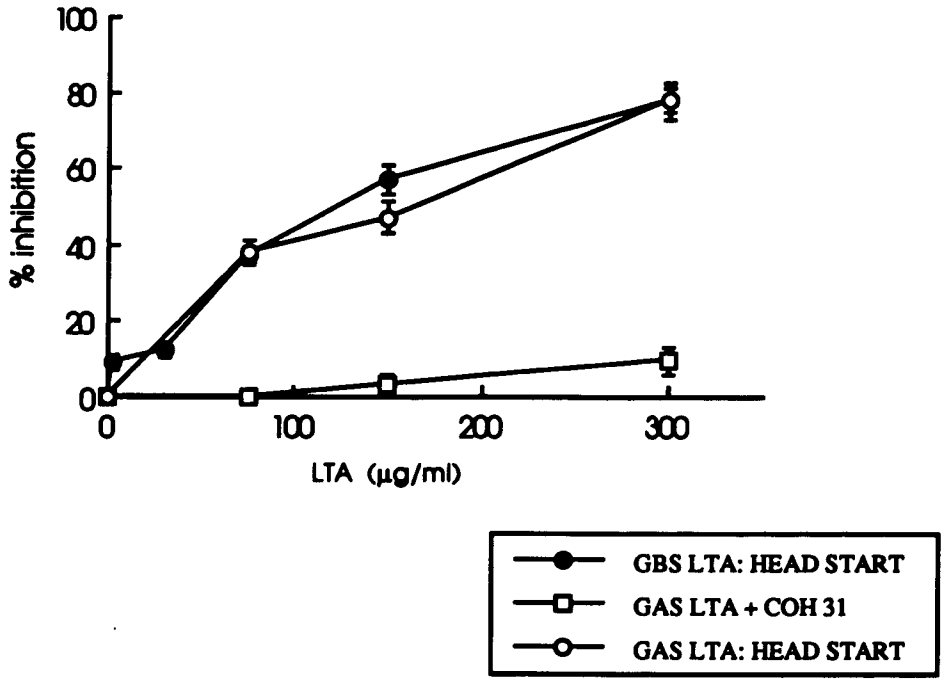

Fig. 6. Inhibition of bacterial binding to Mø by lipoteichoic acid

group A streptococcal (GAS) LTA was tested. GBS binding by macrophage monolayers pre-exposed to this LTA were compared with monolayers exposed to LTA only during the incubation with bacteria. When macrophages are preincubated ('head-start') with GAS-LTA, there was a greater effect on inhibition of bacterial binding than when macrophages were exposed to LTA only in the presence of bacteria. A second study showed a similiar dose-dependent inhibitory effect on the binding of GBS when the macrophages were pre-exposed to LTA from GBS as well (Fig.6).

To further examine the role of LTA in the binding of GBS, macrophages were allowed to adhere to glass or plastic surfaces coated with LTA from group B streptococci. By analogy with the downmodulation studies using monoclonal antibodies to CR3, specific receptors for LTA would be expected to diffuse to the substrate-attached portion of the macrophage, where they are trapped by interaction with the surface-bound LTA, leaving the apical surface of the cells devoid of LTA receptors. As shown in Fig. 7 GBS binding to macrophages was inhibited to a high degree (>60%) when either glass or plastic slides were coated with LTA at concentrations of 30 μg/ml or greater, suggesting the existence of a receptor on the macrophage that recognizes LTA.

When flow cytometry was used to observe the effects of LTA on the ability of macrophages to bind GBS, I obtained results that were consistent with the previous downmodulation and inhibition studies. Figure 8 is an overlay of fluorescence histograms comparing the FSC with fluorescence intensity for

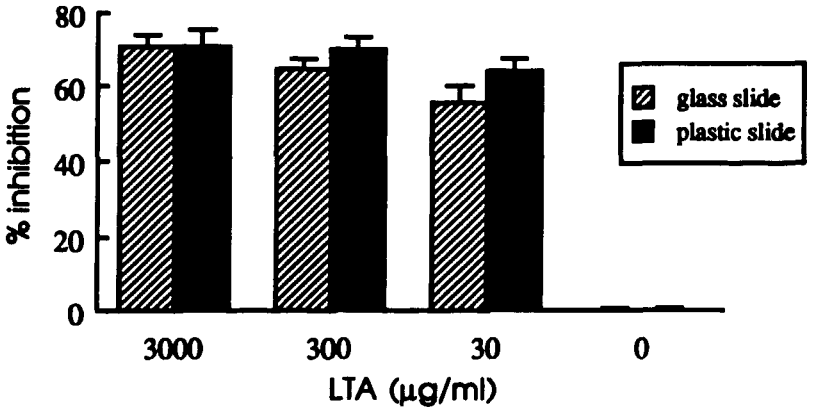

Fig. 7. Effect of downmodulation of LTA receptors on bacterial binding by Mø

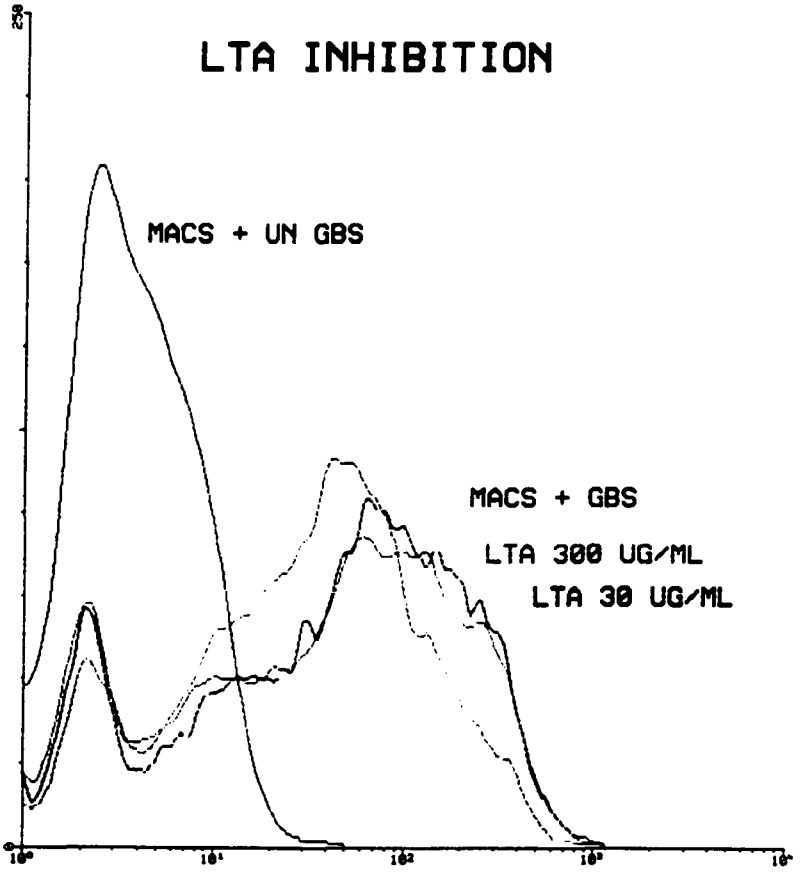

Fig. 8. Effect of lipoteichoic acid on adherence of LY-labeled GBS and Mø. Overlay of histograms for controls: Mø + unlabeled GBS demonstrates inherent fluoresecence of Mø; Mø + LY-labeled GBS demonstrates maximum fluorescence intensity without LTA.

unstained GBS binding to macrophages with maximum fluorescence of LY-labelled GBS binding to macrophages as well as the effect of LTA on the fluorescence intensity of the GBS-bound macrophage. The D/s(n) statistic for LTA at 30 μg/ml was 1.17, indicating no significant difference from the untreated system. Significant inhibition of GBS binding to macrophages, however, was observed for a concentration of LTA at 300 μg/ml (D/s(n) = 10.49). These data are consistent with downmodulation and soluble inhibition studies using LTA, supporting the role of LTA in streptococcal recognition by the macrophage.

Discussion

Macrophages can recognize group B streptococci in the absence of IgG and complement proteins. I show here that a family of three structurally related receptors on macrophages, CR3, LFA-1 and p150,95, are involved in recognizing group B streptococci. When a monoclonal antibody, M1/70, that recognizes the α-subunit of CR3 was added to macrophages or used to downmodulate or ligate CR3 on macrophages, the binding of GBS was reduced (Table 3). The binding of GBS may be reduced to a greater degree when a monoclonal antibody, M18/2 that recognizes the β-subunit, common to all members of the family, is used in these same studies. The monoclonal antibody 5C6, that recognizes a different epitope from M1/70 was not inhibitory. These results

suggest two possibilities for the GBS receptor on macrophages. Either the receptor is CR3 and the binding site is located near the epitope recognized by M18/2 on the β-subunit or all three members of the β_2 family of integrins, CR3, LFA-1 and p150,95, are involved. This mechanism of recognition may thus serve in the initial response of cells to bacteria, before the generation of specific immunity. Consistent with this view are the observations on several patients whose cells lack the common β-chain of CR3, LFA-1 and p150,95 and thus express none of the mature $\alpha_x\beta_2$ dimers on their cell surface (26). These patients suffer recurrent infections (73).

Wright and Jong (116) have shown that each of the three structurally related receptors on macrophages, CR3, LFA-1 and p150,95, is capable of recognizing *E. coli*. Their observations suggest that the receptors must bind the hydrophilic portion of lipopolysaccharide that extends from the *E. coli* membrane. Since the hydrophilic protion of lipid IVa is composed of diglucosamine bisphosphate, the authors suspect that a disaccharide or sugar phosphate provides the structure that is recognized. It is possible that the three proteins, CR3, LFA-1, and p150,95, each recognize subtly different residues in order to provide a greater spectrum of target molecules. Talamás-Rohana *et al.* (98) have shown that the abundant surface glycolipid, lipophosphoglycan (LPG) from *Leishmania mexicana* promastigotes directly binds to members of the CR3, LFA-1, and p150.95 family of leukocyte integrins by the "LPS" binding site. Bullock and Wright (12) have shown that all three

member of the CR3, LFA-1 and p150,95 family share the ability to recognize *Histoplasma capsulatum*. Five novel phosphoinositol-sphingolipids have been purified from the yeast phase of *H. capsulatum* (8,9). The sphingolipids possess an identical inositol phosphoceramide core. Thus the ligand on *H. capsulatum*, the phosphosugar of its sphingolipids, may also be recognized by the "LPS" binding site of the leukocyte integrins.

Experiments by Ross*et al.* (74) suggest that CR3 expresses a lectin-like binding site that is distinct from the C3bi-binding site on the α-subunit. This lectin-like binding site may be located on the shared β-subunit, since all three member of this integrin family bind to *H. capsulatum*. Monosaccharides, β-glucan and yeast mannan failed to block the binding of *H. capsulatum* to macrophages (12). In my own previous studies (see Chapter 2) using monosaccharides and neoglycoconjugates of bovine serum albumin I was unable to block the binding of GBS by mouse peritoneal macrophages. These studies suggest the role of phosphosugars as candidates for the lectin-like recognition site.

Membrane teichoic acid from group B streptococci, lipoteichoic acid (LTA) binds to a trypsin-sensitive glycoprotein receptor on human adult buccal epithelial cells due to specific interactions with its glycerolphosphate backbone (56). My studies on the binding of GBS by macrophages strongly suggest that the ligand on GBS that is recognized by the macrophage receptor is lipoteichoic acid. When macrophages are exposed to soluble LTA prior to GBS, the

binding of bacteria is reduced. Further, LTA-coated surfaces downmodulate the capacity of macrophages to bind GBS .

The site on LTA that is recognized by the macrophage receptor has not been determined. I might presume that because the receptors bind to LTA that is inserted into GBS membranes, the receptors must bind to the hydrophilic portion of the molecule that is composed of sugar phosphates. This would be consistent with the binding of other bacterial phosphosugar surface structures by the leukocyte integrins (12, 77, 98, 116). My studies suggesting the role of LTA and the β_2 integrins in the binding of GBS by murine peritoneal macrophages offer additional support for the role of this family of receptors in the recognition of extracellular pathogens.

OVERALL DISCUSSION

The receptors and ligands involved in mediating non-opsonic adherence of group B stretococci by murine peritoneal macrophages have been explored previously, but not identified. The overall goal of this study was to identify and characterize the receptor on the macrophage responsible for recognition of the bacteria. My premise is that macrophages are at times responsible for cellular defenses in the absence of serum factors such as complement components, and before the induction of specific recognition molecules, antibodies. Macrophages therefore, must have the ability to distinguish self from non-self, independent of soluble molecules. For these studies it was necessary to develop adherence assays that could quantify adherence of GBS to murine macrophages in order to test various substances for their role as inhibitors of binding. I developed two adherence assays, one visual and the other an enzyme-linked immunosorbent assay. Adherence results from these two assays correlate well. These adherence assays as well as those of others show that bacteria do indeed adhere to macrophages under a variety of *in vitro* conditions, suggesting the presence of membrane structures on these cells with the ability to recognize complementary structures on the bacterial surface. Two groups of macrophage membrane receptors have been implicated in microbial recognition, cell-associated lectins, such as the galactose

receptor, and the integrins. The strategy of this study was to determine if either or both of these molecules was involved in bacterial recognition.

Using the adherence assays developed in Chapter 1, I investigated the role of the galactose receptor lectin on the macrophages for binding GBS. Methods comparing isogenic strains of GBS with exposed galactose, neuraminidase–treated bacteria which exposed capsular galactose, and inhibition studies using soluble galactose, BSA-glycoconjugates of galactose as well as other monosaccharides failed to prove the involvement of the macrophage galactose lectin. The stringency of my assay methods and the exhaustive testing using different forms of GBS encourages my findings to dispute other investigators who have shown the attachment of GBS via the lectin receptor for galactose on the macrophage surface.

I then showed that the mechanism for binding of GBS by macrophages, such as temperature (118) and divalent cation dependence (91), protease sensitivity (100), fibronectin and phorbol ester enhancement (115) and 2-deoxy-D-glucose inhibiton (97) were similiar to those of integrin molecules, especially CR3. The presence of monoclonal antibodies to CR3, in downmodulation studies, or as soluble competitors for GBS and macrophage monolayers, using the visual assay, or in macrophage suspensions detected by flow cytometry yielded similiar degrees of inhibition which would suggest the involvement of the receptor for complement component C3bi in the recogni-

tion of GBS.

Talamas-Rohana,*et al.* (98) have described two classes of ligands for CR3. C3bi and fibrinogen possess the peptide RGD (Arg-Gly-Asp) recognized by the α-subunit. The *Leishmania* lipophosphoglycan and *E. coli* lipopolysaccharide are recognized by a separate site on CR3 (116). The inability of MAb M1/70, which recognizes the C3bi binding site, to block adherence as effectively as MAb M18/2, which recognizes the β-subunit (CD18) common to all members of the leucocyte integrin family of receptors, indicates that attachment of GBS may involve a bacterial ligand similiar to the phosphosugars of *Leishmania* and *E. coli*. My studies using purified membrane teichoic acid from GBS, lipoteichoic acid, as an effective inhibitor of macrophage adherence suggest its role in the adherence of GBS via a site on CD18.

Macrophages recognize GBS in the absence of IgG, complement proteins, or bacterial proteins. This novel mechanism of recognition of a gram-positive, extracellular pathogen may thus serve as an initial response of cells to bacteria, before the generation of specific immunity. Consistent with this view are the observations on several LAD patients whose cells lack the common β-subunit of CR3, LFA-1 and p150, 95 and express none of the mature $\alpha_x\beta_2$ on their cells surface (91). These patients suffer from recurrent bacterial infections. The basis for the susceptibility of neonates as compared with adults to GBS infection still remains an area for speculation. The

predominant hypothesis for this observation is based on evidence of decreased complement levels and function in neonates vs. adults (23). Reduced levels or function of CR3 in neonatal vs. adult macrophages might explain the differential susceptibility of neonates to bacteria whose uptake is CR3 dependent.

Note in added proof.

In a recent study by Antal *et al.* (2), the authors report the role of CR3 in the opsonin-independent phagocytosis of heat-killed group B streptococci by the mouse macrophage-like cell line, PU5-1.8 (monocytic tumor, BALB/c) and resident peritoneal macrophages from BALB/c mice. Downmodulation of surface Mac-1 (CR3) on macrophages following adherence to anti-Mac-1- (M1/70) or anti-CD18- (M18/2) coated surfaces inhibited uptake of GBS, scored by microscopic examination. Soluble anti-Mac-1 inhibited phagocytosis of GBS by as much as 50% for both mouse peritoneal macrophages and the macrophage cell line, whereas soluble anti-CD18 had no effect.

These data support our own studies with one exception. We report that soluble M18/2 (anti-CD18) inhibits the binding of GBS to murine peritoneal macrophages almost twice as effectively as soluble M1/70 (anti-Mac-1), suggesting that binding site might be on the β-subunit and GBS adherence may be relevant for all members of the leukocyte family of integrins. This

result may be explained by differences in the cell surface of resident vs. thioglycollate-elicited peritoneal macrophages and between heat-killed and live bacteria.

Our studies using LTA to inhibit adherence of GBS to macrophages suggests a major role for the lectin site on the β-subunit leukocyte integrins for the binding of GBS consistent with other studies demonstrating adherence of *E. coli* (116) and *Leishmania* (98), through their phosphosugars, LPS and LPG, respectively. Antal *et al.* indicate (no data shown) that soluble anti-CD18 inhibits GBS uptake by human cells but not mouse cells, supporting the role of the β-subunit in bacterial adherence. In this recent study, using mouse macrophages, the authors postulate that inhibited phagocytosis of GBS may involve blocking of a recognition site on CR3 by anti-Mac-1 or may involve some antibody-induced inhibition of a general signalling functon of CR3 in phagocytosis. Their study does not clearly differentiate these possibilities.

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