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Opsonin-Independent Phagocytosis of Group B Streptococci: Role of Complement Receptor Type Three

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The role of complement receptor type 3 (CR3) in nonopsonic recognition of group B streptococci (GBS) by macrophages was investigated. Monoclonal anti-CR3 (anti-Mac-1) inhibited phagocytosis of GBS strains by as much as 50% in serum-free cultures of both mouse peritoneal macrophages and the macrophage cell line PU5-1.8. GBS uptake was unaffected by the presence of anti-C3 or salicylhydroxamate, an inhibitor of the covalent binding reaction of C3. Soluble antibodies to LFA-1 or to the common beta-chain (CD18) of the LFA-1/CR3/p150,95 family of cell adhesion molecules did not inhibit GBS uptake. Down-modulation of surface Mac-1 on macrophages following adherence to anti-Mac-1- or anti-CD18-coated surfaces also inhibited uptake of GBS. Further evidence for GBS interaction with CR3 was demonstrated by reduction of EC3bi rosette formation in macrophages adherent to GBS-coated plates. These studies suggest that GBS can interact with macrophage CR3, promoting phagocytosis in a C3-independent fashion. In the absence of specific immunity in neonates, this recognition mechanism may be a significant virulence determinant for GBS which poorly activate the alternate complement pathway.

Recent evidence shows that the CD11/CD18 family of leukocyte adhesion molecules (LFA-1, complement receptor type 3[CR3], p150,95) mediates C3-independent binding and uptake of a variety of microbes and microbial cell wall components such as *Escherichia coli* (45), lipopolysaccharide (46), *Staphylococcus epidermidis* (37), *Leishmania* species (27), *Leishmania* glycoprotein gp63 (38) and lipophosphoglycan (43), *Histoplasma capsulatum* (9), beta-glucan (36), and *Bordetella pertussis* hemagglutinin (32). The LFA-1/CR3/p150,95 molecules found on the surface of phagocytes are structurally homologous alpha-beta-chain heterodimers composed of 150- to 190-kDa antigenically distinct alpha chains (CD11a,b,c) and 95-kDa identical beta chains (CD18, -3). Binding of such ligands to the phagocyte CR3 is inhibited by antibodies to the alpha chain of CR3 directed at the C3bi site (recognizes Arg-Gly-Asp or similar sequences [38, 46]), antibodies directed to lectinlike sites (35, 36), or antibodies to the leukocyte adhesion molecule common beta chain (37). Thus, CR3 is implicated to possess multiple sites through which inflammatory microbes may bind, either directly or via fixed C3, and activate phagocyte functions (11, 35). Complement receptor type four (CR4 [p150,95]) may also possess similar ligand-binding sites (9, 28, 45).

Group B streptococci (GBS) are major etiological agents of neonatal meningitis and septicemia (18). In the absence of specific antibody and with limited complement-mediated opsonization of GBS in neonates (16, 18), susceptibility of GBS to intrinsic opsonin-independent phagocytosis becomes an important virulence determinant. Antibody- and complement-independent uptake of type I, II, and III GBS has been described in both human neutrophils and mouse macrophages (2, 6, 24, 44). GBS also interact with cultured macrophages in the absence of antibody opsonins to stimulate enhanced C3 synthesis (20). The molecular basis of nonopsonic recognition between GBS and phagocytes is largely unknown.

Experiments described here examine the role of CR3 in antibody- and complement-independent interactions of GBS with cultured macrophages. Monoclonal anti-CR3 (anti-Mac-1) was found to inhibit GBS uptake by macrophages. Phagocytosis of GBS strains by mouse macrophages and macrophage cell lines in serum-free cultures was found to be CR3 dependent but C3 independent.

MATERIALS AND METHODS

Macrophages. The mouse macrophagelike cell line, PU5-1.8 (monocytic tumor, BALB/c [31]), was obtained from the American Type Culture Collection (Rockville, Md.). PU5-1.8 cells were maintained in Dulbecco modified Eagle medium with 4,500 mg of glucose (GIBCO) per liter supplemented with 10% (vol/vol) fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) and antibiotic-antimycotic (GIBCO) in a humidified atmosphere with 5% CO_2 at 37°C. When grown to confluence, these cells were subcultured by gentle agitation and dilution into fresh medium.

Resident peritoneal macrophages were obtained by peritoneal lavage of CO_2-asphyxiated BALB/c mice (male or female, bred in-house or purchased from Charles Rivers Laboratories) with 3 to 5 ml of cold Dulbecco calcium- and magnesium-free phosphate-buffered saline (PBS) plus 2 mg of glucose per ml. Macrophage numbers were determined based on total and differential cell counts of peritoneal cells. Macrophages were purified by adherence to 96-well culture plates (10^5 macrophages per well) or chamber slides (Lab-Tek; Nunc, Inc.; 2×10^5 macrophages per well) for 90 to 120 min; this was followed by washing to remove nonadherent cells.

Streptococci. GBS (serotype Ia, strains 090, DS1204, 509-80; serotype III, strains D136C, M732, 603-79,2871) were grown in a modified Todd-Hewitt broth (5) overnight at 37°C. Strains DS1204 and M732 were kindly provided by Toby Eisenstein (Temple University, Philadelphia, Pa.). Strain

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2871 was kindly provided by Mario Marcon (Children's Hospital, Columbus, Ohio). Bacteria were harvested by centrifugation (10,000 × *g*, 15 min) and washed three times in PBS. Cell numbers were determined by viable colony counts on sheep blood agar plates. Cell suspensions were heat killed (60°C, 30 min) and were stored at 4°C. Fluorescein isothiocyanate-labeled GBS were prepared as described by Wright and Jong (45).

Antibodies. $F(ab')_2$ goat anti-mouse C3 and $F(ab')_2$ goat immunoglobulin G (IgG) were purchased from Cappel/Organon Teknika (Durham, N.C.). Commercial monoclonal anti-Mac-1 and anti-Mac-3 were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Monoclonal antibodies to macrophage surface antigens were also used as culture supernatant fluids (serum-free Dulbecco modified Eagle medium) or as affinity-purified (anti-rat IgG or anti-mouse IgG Sepharose 4B) antibodies from the following hybridoma cell lines: M1/70.15.11.5 (rat IgG2b, anti-Mac-1 or CD11b), M3/84.6.34 (rat IgG1, anti-Mac-3 antigen), M17/4.2 (rat IgG2a, anti-mouse LFA-1 alpha or CD11a), M18/2.a.8 (rat IgG2a, anti-mouse LFA-1 beta or CD18), 2.4G2 (rat IgG2b, anti-mouse Fc receptor type II [FcRII]), TS1/22.1.1.13 (mouse IgG1, anti-human LFA-1 alpha or CD11a), TS1/18.1.2.11 (mouse IgG1, anti-human LFA-1 beta or CD18), 543.2 (mouse IgG1, anti-human CR1). Hybridoma 2.4G2 was kindly provided by Jay Unkeless (Mount Sinai School of Medicine, New York, N.Y.). All other hybridomas were obtained from the American Type Culture Collection. Anti-Mac-1 and anti-CD18 were biotinylated with sulfo-NHS-biotin per the manufacturer's directions (Pierce Chemical Co., Rockford, Ill.).

Phagocytosis assay. Adherent macrophages were cultured overnight, washed twice, and placed in serum-free Dulbecco modified Eagle medium. Antibodies, when appropriate, were directly added to the medium. Antibodies were added as serum-free culture supernatant fluids or in purified form in PBS and were present throughout the incubation with GBS. One hour later, in the presence or absence of antibody, GBS or latex beads (1.1 μm; Sigma) were directly added at a ratio of 10:1 or 100:1 (GBS or bead/macrophage). Salicylhydroxamate (Sigma) was added to some cultures to inhibit endogenous complement-mediated opsonization. Cultures were then incubated for 3 h, and adherent macrophages were washed to remove nonassociated bacteria. The cells were fixed and stained with the Leukostat stain kit (Fisher Scientific Co., Pittsburgh, Pa.). Phagocytosis and binding were scored by microscopic examination as the number of macrophages with more than one streptococcus or bead per total number of macrophages counted (percent phagocytosis) and as the number of bacteria engulfed per 100 macrophages (phagocytic index). A minimum of 100 macrophages per sample were examined.

To differentiate attached versus ingested GBS, we measured phagocytosis of fluorescein-labeled GBS. Internalized bacteria were distinguished from extracellular bacteria by either quenching extracellular fluorescence with methylene blue (39) or shifting the fluorescence of extracellular bacteria from green to red by the addition of ethidium bromide at the end of the assay (12).

Antibody-coated culture plates. Conditioned Dulbecco modified Eagle medium or purified antibody (100% serum-free hybridoma supernatant fluid or 50 μg of purified antibody per ml, 0.05 ml per well) was coated onto 96-well tissue culture wells or on glass Lab-Tek slides by incubation for 1 h at room temperature (RT). To enhance antibody binding, surfaces were incubated with poly-L-lysine (Sigma; 365,000 molecular weight; 0.1 mg/ml in PBS, 30 min, RT), washed, and then treated with 2.5% glutaraldehyde in PBS (15 min, RT). Treated surfaces were washed extensively with distilled water and then with PBS prior to the addition of antibody coating for 1 h. Antibody-coated surfaces were washed, and unreacted aldehyde groups were quenched for 1 h by the addition of 0.1 M glycine–1% bovine serum albumin (BSA) in 0.01 M phosphate buffer (pH 7.5). Antibody-coated surfaces were washed in PBS prior to adherence of macrophages for 2 h.

GBS-coated culture plates. GBS were fixed to microtiter plates by a modified method of Polin and Kennett (29). Briefly, 0.05 ml of poly-L-lysine (0.001 g/100 ml of PBS, pH 7.2) was added to each well of a 96-well flat-bottomed plate and incubated for 2 h at RT. Poly-L-lysine was removed, and GBS suspensions were added to each well. Culture plates were centrifuged in a microwell plate carrier (830 × *g*. ɔ min), and 0.5% glutaraldehyde in cold PBS was added (0.25% [vol/vol] final concentration) directly to each well. Following a 15-min incubation at RT, all wells were washed twice with PBS.

Macrophage surface antigen assay. Surface antigens of control or treated macrophages were assessed by an enzyme-linked immunosorbent assay (ELISA) on fixed cells. Adherent macrophages (10^5 per well of 96-well flat-bottomed plates) were washed with PBS and fixed by the addition of 0.25% glutaraldehyde in PBS for 15 min at RT. This fixation procedure completely preserves Mac-1 reactivity with antibody and preserves antigenicity of most other macrophage antigens as well (23). Fixed cells were washed with PBS and blocked with 1% BSA in PBS for 30 min. Purified antibodies (0.05 ml per well) were added at 10 μg/ml in 1% (wt/vol) BSA in PBS. Hybridoma supernatant fluid was added at 100% (vol/vol). Following a 60- to 90-min incubation at RT, the plate was washed three times with 0.25% BSA-PBS with 0.05% Tween 20. Secondary antibody, peroxidase-conjugated goat anti-rat Ig (Southern Biotechnology Associates, Inc., Birmingham, Ala.), was diluted 1:1,000 in 1% BSA-PBS, and 0.05 ml per well was added. Following a 1-h incubation and three washes as above, 0.05 ml of enzyme substrate solution (0.2% *o*-phenylenediamine in 17 mM citrate–65 mM phosphate buffer [pH 6.0] plus 0.015% hydrogen peroxide) was added per well and incubated for 15 min at RT in the dark. Reactions were stopped by the addition of 0.05 ml of 12.5% sulfuric acid per well. A_{490} was measured with an automated microplate reader (Bio-Tek Instruments, Winooski, Vt.).

For ELISA of surface antigens on macrophages adherent to rat antibody-coated surfaces, biotinylated anti-Mac-1 and anti-CD18 were used as primary antibodies followed by staining with peroxidase-conjugated streptavidin (Zymed Laboratories, South San Francisco, Calif.). For ELISA of other antigens, background binding of the secondary antibody (peroxidase-conjugated anti-rat IgG) alone to antibody-adherent cell monolayers were subtracted from the binding reaction of cells reacted with both primary and secondary antibodies. The cell monolayers masked most of the background binding of the secondary antibody to the coated antibodies so that this method could distinguish the coated antibody from the staining antibody.

EC3bi. EC3bi (erythrocyte-antibody complement complexes) used to detect functional CR3 on macrophages were prepared by the sequential addition of anti-sheep erythrocytes (IgM) and complement (C5-deficient mouse serum) to sheep erythrocytes (34). EC3bi preparations were free of EC3b complexes as shown by absence of rosette formation

TABLE 1. Nonopsonic phagocytosis of GBS by macrophages

GBS strain	% Phagocytosis[a]	
	PU5-1.8 cells	Resident mouse macrophages
Type Ia		
090	35 ± 5 (6)	48 ± 2 (3)
DS1204	26 ± 7 (3)	ND[b]
509-80	24 ± 15 (3)	ND
Type III		
M732	18 ± 4 (3)	21 ± 3 (2)
D136c	19 ± 7 (3)	ND
603-79	16 ± 6 (3)	ND

[a] Phagocytosis was scored microscopically as percentage of total macrophages with engulfed bacteria 3 h after addition of heat-killed streptococci (10 GBS per macrophage) to adherent macrophages. Values represent mean ± standard deviation of one experiment. The number of samples per experiment is given in parentheses. Similar data were found in replicate experiments.
[b] ND, not determined.

with human erythrocytes (34). Rosetting assays were performed as described by Ross (34). EC3bi rosettes with macrophages were scored microscopically, and a rosette was defined as a macrophage with at least three erythrocytes attached. The results were expressed as percent rosetting, or the number of macrophages rosetted per total number of macrophages counted (minimum of 100).

Statistics. Significance between means of experimental groups was determined by the Mann-Whitney U test, Wilcoxon signed-rank test, or Student's *t* test.

RESULTS

Phagocytosis of GBS in serum-free macrophage cultures. Phagocytosis of GBS strains was evident in 20 to 50% of macrophages. A comparison of GBS strains for nonopsonic phagocytosis by PU5-1.8 and resident mouse peritoneal macrophages (Table 1) suggested that higher percentages of macrophages engulfed representative type Ia strains than type III strains; however, phagocytic indices were not compared. Phagocytosis experiments with fluoresceinated GBS in which methylene blue or ethidium bromide was used to differentiate extracellular from intracellular bacteria confirmed that phagocytosis values represented internalized GBS. A representative experiment found 51% ± 1% phagocytosis of fluorescein isothiocyanate-labeled GBS strain 090 by resident peritoneal macrophages, which resembled values determined by microscopic examination of stained cells.

Role of Mac-1 in phagocytosis of GBS. Mouse macrophage phagocytosis of GBS was inhibited by 30 to 50% by treatment of adherent macrophages with anti-Mac-1, while it was not significantly inhibited by treatment with anti-LFA-1 or by treatment with anti-CD18 (Table 2). Results were similar whether measuring uptake by fluorescence microscopy or by microscopic examination of fixed and stained cells. Summarizing data from several experiments, anti-Mac-1 inhibited phagocytosis of GBS 090 by 31% in resident macrophages (49% inhibition when judging phagocytic indices). Anti-Mac-1 also inhibited phagocytosis of 090 by 44% in thioglycolate-elicited inflammatory macrophages and by 63% in *Corynebacterium parvum*-activated macrophages (data not shown). Dose-response studies revealed progressive inhibition of phagocytosis with increasing final concentrations of M1/70 conditioned medium or purified anti-Mac-1, but no more than 50 to 60% inhibition of phagocytosis could be achieved at any concentration of anti-Mac-1, but no more than 50 to 60% inhibition of phagocytosis could be achieved at any concentration of anti-Mac-1. Purified anti-Mac-1 (1 to 10 μg/ml) also inhibited phagocytosis in PU5-1.8 cells maximally to approximately 50% of controls. Anti-Mac-1 did not cause a general inhibition of phagocytosis as shown by uninhibited uptake of latex beads [104% ± 22% (n = 3) and 94% ± 10% (n = 2) of untreated control-level phagocytosis for PU5-1.8 and resident peritoneal macrophages, respectively]. Anti-Mac-1 effects were dependent on treating the macrophages since pulse treatment of bacteria with anti-

TABLE 2. Macrophage phagocytosis of GBS 090: inhibition by anti-Mac-1

Antibody treatment[a]	Phagocytosis (% of control)[b]	Phagocytic index (% of control)[b]
Resident peritoneal cells		
None	100 ± 11	100 ± 17
Anti-Mac-1	69 ± 16 (11) ($P < 0.05$)	53 ± 11 (6) ($P < 0.05$)
Anti-CD18	106 ± 26 (5)	116 ± 46 (3)
Anti-LFA-1 (CD11a)	103 ± 35 (3)	252 (1)
ANti-FcRII	114 ± 25 (2)	149 (1)
PU5-1.8 cells		
None	100 ± 36	100 ± 12
Anti-Mac-1	51 ± 13 (13) ($P < 0.05$)	47 ± 5 (3) ($P < 0.05$)
Anti-CD18	95 ± 4 (3)	108 (1)
Anti-LFA-1 (CD11a)	85 ± 5 (2)	ND[c]
Anti-Mac-3	106 ± 14 (3)	ND
Anti-FcRII	88 ± 3 (4)	ND
Anti-FcRII + anti-Mac-1[d]	53 ± 8 (2) ($P < 0.05$)	ND

[a] Hybridoma-conditioned medium (50% [vol/vol], final concentration) or purified antibodies (10 μg/ml) were added to serum-free macrophage cultures 1 h prior to addition of GBS.
[b] Phagocytosis was assessed 3 h after the addition of 10 or 100 GBS per macrophage and is expressed as percentage of control values in non-antibody-treated macrophages. Absolute values for phagocytosis in resident macrophages were 32 ± 9 (10 GBS per cell, five experiments) and 63 ± 7 (100 GBS per cell, seven experiments) for percent phagocytosis and 77 ± 9 (10 GBS per cell, four experiments) and 248 ± 43 (100 GBS per cell, eight experiments) for phagocytic index. Absolute values for phagocytosis in PU 5-1.8 cells were 61 ± 22 (10 GBS per cell, seven experiments) for percent phagocytosis and 33 ± 4 (10 GBS per cell, three experiments) and 84 (100 GBS per cell, one experiment) for phagocytic index. Number in parentheses shows number of experiments (two or more samples per group per experiment). Values express means ± standard deviation.
[c] ND, not determined.
[d] Anti-FcRII (10 μg/ml) was added 30 min prior to addition of anti-Mac-1 to the same culture.

TABLE 3. Phagocytosis of GBS by human leukocytes: inhibition by anti-Mac-1

Antibody treatment[a]	Phagocytosis (% of control)[b]	Phagocytic index (% of control)[b]
Neutrophils		
None	100 ± 15 (3)	100 ± 17 (3)
Anti-Mac-1	39 ± 11 (3)[c]	26 ± 7 (3)[c]
Anti-CD18	23 ± 9 (3)[c]	15 ± 6 (3)[c]
Anti-LFA-1	94 ± 10 (3)	94 ± 10 (3)
Anti-CR1	100 ± 1 (3)	100 ± 1 (3)
Monocytes		
None	100 ± 20 (3)	100 ± 24 (3)
Anti-Mac-1	43 ± 10 (3)[c]	37 ± 10 (3)[c]
Anti-CD18	53 ± 14 (3)[c]	40 ± 13 (3)[c]
Anti-LFA-1	80 ± 4 (3)	83 ± 15 (3)
Anti-CR1	94 ± 9 (2)	91 ± 13 (2)

[a] Purified antibodies (5 μg/ml) were added 30 min prior to GBS.
[b] Peripheral blood monocytes and neutrophils were isolated from heparinized blood by centrifugation through Histopaque gradients (Sigma), washed, and suspended in Hank's balanced salt solution. Phagocytosis was assessed 3 h after the addition of 20 GBS per leukocyte with type III GBS strain 2871 and is expressed as percentage of control values in non-antibody-treated leukocytes. Number in parentheses shows number of experiments (two or more samples per group). Values express means ± standard deviation.
[c] Significantly different ($P \leq 0.05$) from untreated control.

TABLE 4. Effect of C3 antagonists on phagocytosis of GBS strain 090 by macrophages in serum-free culture

Treatment[a]	Phagocytosis of 090	
	% Phagocytosis[b]	% Inhibition[c]
Resident peritoneal macrophages		
None	86 ± 5	0
Anti-Mac-1	58 ± 0 ($P < 0.05$)	33
Anti-C3, $F(ab')_2$	83 ± 2	3
Goat IgG, $F(ab')_2$	77 ± 1	11
Salicylhydroxamate	86 ± 1	0
PU5-1.8 (expt 1)		
None	83 ± 1	0
Anti-Mac-1	61 ± 3 ($P < 0.05$)	26
Salicylhydroxamate	78 ± 3	6
PU5-1.8 (expt 2)		
None	71 ± 1	0
Anti-C3, $F(ab')_2$	77 ± 4	0

[a] Anti-Mac-1 (50% [vol/vol] conditioned medium), $F(ab')_2$ anti-C3 (10 μg/ml), $F(ab')_2$ goat IgG (10 μg/ml), and salicylhydroxamate (5 mM) were added to macrophages 1 h prior to addition of bacteria.
[b] Phagocytosis was measured 3 h after the addition of bacteria (10 GBS per macrophage) and is expressed as mean ± standard deviation ($n = 2$) of single experiments representative of replicate experiments.
[c] Percent inhibition of control levels of phagocytosis.

Mac-1 prior to their addition to macrophage cultures did not inhibit uptake. Anti-Mac-1 effects were not dependent on interaction via FcR or due to nonspecific isotype (rat IgG2b) effects since the isotype-matched anti-FcRII did not inhibit macrophage uptake of GBS nor did pretreatment of macrophages with anti-FcR inhibit the effects of anti-Mac-1 (Table 2). Treatment of macrophages with an antibody (conditioned medium [CM] or 1 to 10 μg of purified antibody per ml) to an unrelated surface marker (Mac-3) also did not inhibit uptake of GBS and marginally enhanced phagocytosis in some experiments. Anti-Mac-1 and anti-human CD18 also significantly inhibited phagocytosis in both human monocytes and neutrophils (Table 3). Neither anti-human LFA-1 or anti-human CR1 inhibited GBS phagocytosis.

Role of C3 in phagocytosis of GBS. To assess the contribution of endogenously generated C3b or C3bi in macrophage phagocytosis of GBS, we added $F(ab')_2$ anti-C3 to macrophage cultures in quantities sufficient to bind all endogenously secreted C3 (mouse C3 not detectable by ELISA in culture medium from anti-C3-treated cultures) and sufficient to inhibit EC3bi rosetting (resident macrophages did not form EC3bi rosettes in the presence of anti-C3). Whereas anti-Mac-1-treated macrophages had inhibited GBS uptake compared with untreated controls, $F(ab')_2$ anti-C3-treated macrophages exhibited no inhibition of GBS uptake compared to control cultures treated with $F(ab')_2$ goat IgG (Table 4). Furthermore, salicylhydroxamate, an inhibitor of the covalent binding reaction of C3b (40), added directly to serum-free cultures of PU5-1.8 or resident macrophages at a dose (5 mM) sufficient to inhibit classical complement-mediated hemolysis did not affect uptake of GBS 090 (Table 4). These data suggest that endogenous cultural C3 and complement fixation were not contributing to the phagocytosis of GBS.

PU5-1.8 cells and resident peritoneal macrophages exhibited small increases in phagocytosis (12 and 18%, respectively; data not shown) and increased numbers of engulfed GBS per macrophage with serum-opsonized (pooled fresh mouse serum) GBS versus unopsonized GBS or GBS treated with heat-inactivated serum. Unopsonized GBS and GBS incubated with salicylhydroxamate-treated mouse serum were engulfed equally by PU5-1.8 cells. Anti-Mac-1 treatment of macrophages reduced phagocytosis of unopsonized and serum-opsonized GBS to the same final levels, indicating that phagocytosis of GBS, unopsonized or complement opsonized, is mediated via CR3.

Phagocytosis of GBS by macrophages depleted of CR3 by adherence to anti-Mac-1. Specific down-modulation of surface Mac-1 has been reported to occur on macrophages adherent to specific antibody-coated surfaces (47). PU5-1.8 cells and resident mouse macrophages were thus adhered to anti-Mac-1-coated surfaces and compared with macrophages adherent to uncoated surfaces for phagocytosis of GBS. Macrophages adherent to anti-Mac-1-coated surfaces exhibited relatively specific down-modulation of surface Mac-1 antigen to less than 50% of control levels in PU5-1.8 and resident macrophages (Tables 5 and 6). Incomplete modula-

TABLE 5. Phagocytosis of GBS 090 by PU5-1.8 cells adhered to anti-Mac-1-coated surfaces

Antibody coating[a]	Mac-1 level (% of control)[b]	Phagocytosis (% of control)[c]
None	100 ± 8 (3)[d]	100 ± 5 (3)
Anti-Mac-1	13 ± 10 (3)	55 ± 8 (3) ($P < 0.05$)
Anti-LFA-1	85 ± 11 (1)	106 ± 1 (1)
Anti-Mac-3	72 ± 13 (1)	97 ± 2 (1)
Anti-FcRII	78 ± 5 (1)	94 ± 4 (1)
Anti-CD18	70 ± 14 (1)	91 ± 3 (1)

[a] Tissue culture wells were coated with 100% (vol/vol) serum-free hybridoma-conditioned medium or purified antibody (50 μg/ml) for 1 h prior to adherence of macrophages to wells for 2 h.
[b] Values were calculated as percentage of control levels (optical densities of developed ELISA) of cells adherent to uncoated or BSA-coated surfaces. Values represent means ± standard deviation.
[c] Values calculated as percentage of control phagocytosis (3 h, 10 GBS per macrophage) in cells adherent to uncoated or BSA-coated surfaces. Values expressed as mean ± standard deviation.
[d] Number of experiments, six samples each.

TABLE 6. Phagocytosis of GBS 090 by mouse resident peritoneal macrophages adhered to anti-Mac-1-coated surfaces

Antibody coating[a]	Antigen level (% of control)[b]			Phagocytosis[c]	
	Mac-1	LFA-1	CD18	%	PI
BSA	100 ± 17	100 ± 29	100 ± 9	41 ± 4	215 ± 20
Anti-Mac-1	32 ± 12	83 ± 29	71 ± 8	33 ± 5[e]	123 ± 37[e]
Anti-Mac-1	50 ± 9*	ND[d]	76 ± 10*	ND	ND
Anti-LFA-1	71 ± 7	51 ± 63	63 ± 12	44 ± 4	191 ± 4
Anti-CD18	8 ± 16	45 ± 32	20 ± 13	42 ± 1	159 ± 6[e]
Anti-Mac-3	143 ± 11	ND	118 ± 8	64 ± 8[e]	337 ± 28[e]
Anti-FcRII	116 ± 11	ND	114 ± 8	46 ± 5	205 ± 40

[a] Purified antibodies (50 μg/ml) were coated on surfaces for 1 h.
[b] Values were calculated as percentage of control levels (optical densities of developed ELISA) of cells adherent (1 h) to BSA-coated surfaces. Values represent means ± standard deviation of six samples of a single experiment representative of replicate experiments. Asterisk (*) shows values obtained with a biotin-streptavidin system (nine samples each).
[c] Values represent means ± standard deviation of four samples from a single experiment representative of replicate experiments. PI, phagocytic index.
[d] ND, not determined.
[e] $P < 0.05$ compared with untreated control.

TABLE 7. EC3bi rosette formation on macrophages adherent to GBS-coated surfaces

Streptococcal coating[a]	EC3bi rosettes (% of control)[b]			
	PU5-1.8		Resident macrophage	
	Expt 1	Expt 2	Expt 1	Expt 2
None (control)	100	100	100	100
Type Ia				
090	36 ± 6	43 ± 0	11 ± 2	57 ± 9
DS1204	42 ± 5	46 ± 6	26 ± 5	ND[c]
509-80	60 ± 3	37 ± 4	14 ± 1	ND
Type III				
M732	76 ± 11	48 ± 10	41 ± 6	ND
D136c	72 ± 8	43 ± 7	19 ± 2	ND

[a] Heat-killed GBS (10^8 CFU per well) were fixed onto poly-L-lysine-coated microtiter culture wells.
[b] Rosettes were scored microscopically on macrophages adherent (2 h) to GBS-coated wells. Values are expressed as the percentage of rosetting seen in control cells adherent to uncoated wells. Means of two samples ± standard deviation of representative experiments are shown.
[c] ND, not determined.

tion of Mac-1 has also been reported by Graham et al. (21). Since LFA-1 and CR3 share the CD18 antigen, macrophages adherent to anti-CD18 would be expected to show reduced Mac-1 and LFA-1, as seen in Table 6. Conversely, macrophages adherent to anti-Mac-1 or anti-LFA-1 would show reductions in CD18 relative to the total ratios of LFA-1, CR3, and p150,95 on their surface. Mac-1 levels were comparatively unaffected or were elevated (anti-Mac-3 adherent resident cells) on macrophages adherent to plates coated with antibodies to distinct macrophage surface antigens. Phagocytosis of GBS in PU5-1.8 cells adherent to anti-Mac-1-coated surfaces was 55% of control values (Table 5). The phagocytic index of PU5-1.8 cells adherent to anti-Mac-1 was 49% ± 16% (three samples) of control cells adherent to uncoated surfaces. Percent phagocytosis and phagocytic index in resident peritoneal macrophages adherent to anti-Mac-1-coated surfaces were 80 and 57% of control values, respectively (Table 6). In replicate experiments, GBS uptake was not significantly affected in macrophages adherent to surfaces coated with BSA, normal rat IgG, anti-LFA-1, or anti-FcR. These antibodies caused little or no down-modulation of Mac-1. Phagocytosis of 090 was decreased only in macrophages with reduced surface Mac-1 following adherence to anti-Mac-1 or anti-CD18. Resident peritoneal macrophages adherent to anti-CD18 (Table 6) showed statistically significant reductions of both apical Mac-1 levels and phagocytic index. The disparity between anti-CD18 effects on phagocytic index versus percent phagocytosis (Table 6) is unexplained; however, in almost all cases in which phagocytosis was reduced by either anti-Mac-1 or anti-CD18, phagocytic index was reduced much more than percent phagocytosis. It may be that modulated Mac-1 has a reduced efficiency of GBS interactions (phagocytic index) but that modulated Mac-1 retains phagocytic function (percent phagocytosis) unless the specific GBS-binding site is blocked. In PU5-1.8 cells (Table 5), in which anti-CD18 did not cause a significant down-modulation of CR3, phagocytosis was not significantly reduced. Resident peritoneal macrophages adherent to anti-Mac-3 exhibited an interesting increase in surface Mac-1 with a corresponding significant increase in both percent phagocytosis and phagocytic index for GBS (Table 6). Phagocytosis of latex beads was slightly elevated in macrophages adherent to anti-Mac-1-coated plates (experiment 1, 30% ± 5% and 44% ± 4% phagocytosis of latex in uncoated versus anti-Mac-1-coated groups, respectively). The inhibitory effects of immobilized anti-Mac-1 and anti-CD18 demonstrate a specific requirement of CR3 for maximal uptake of unopsonized GBS.

GBS inhibition of EC3bi rosettes. Further evidence of GBS interaction with CR3 was displayed when macrophages were adhered to surfaces coated with representative strains of type Ia and type III GBS. Both PU5-1.8 and resident peritoneal macrophages adherent to GBS-coated surfaces demonstrated a loss of functional CR3 as shown by decreased EC3bi formation (Table 7).

DISCUSSION

These studies suggest that antibody and complement-independent phagocytosis of GBS is mediated in part by macrophage CR3 as shown by inhibition with anti-Mac-1. Macrophages adhering to anti-Mac-1- or anti-CD18-coated surfaces had diminished phagocytosis of GBS, and macrophages adhering to GBS-coated surfaces had diminished CR3 function (EC3bi rosettes). A direct recognition event between GBS and CR3 is indicated. Prior to development of specific anti-GBS immunity in newborns infected with GBS, this recognition mechanism may be a significant event in defense against GBS which do not activate the alternate complement pathway.

The importance of leukocyte adhesion molecules (LFA-1, CR3, p150,95) in all aspects of leukocyte function (adherence, migration, particle recognition, phagocytosis, cellular activation) has been revealed in recent years (reviewed in reference 3). This family of molecules can serve as receptors to mediate opsonin-dependent and opsonin-independent binding and phagocytosis of many diverse microorganisms (27, 32, 37, 45). A rare inherited deficiency of leukocyte adhesion molecules, leucocyte adhesion deficiency disease (LAD or Leu-CAM deficiency), results in recurrent and often fatal bacterial infections (3). Observations of impaired leucocyte adhesion molecule levels (8, 19) or function (1) in neonatal cells leads directly to the question of what role

these molecules play in neonatal infections such as GBS infection.

Macrophage phagocytosis of GBS in serum-free conditions was inhibited by anti-Mac-1, a monoclonal antibody specific for the alpha chain of CR3. Antibodies to other leukocyte adhesion molecule antigens (CD11a, CD18) had little or no inhibitory effect when used as soluble treatments of macrophages. Anti-CD18 inhibited GBS phagocytosis only when immobilized on culture surfaces prior to macrophage adherence. These results indicate that the M18 hybridoma-derived anti-CD18 does not recognize or interfere with any putative binding site for GBS on CR3 but can inhibit GBS phagocytosis by down-modulating CR3 on macrophages adherent to anti-CD18. In contrast, the anti-human CD18 antibody inhibited GBS uptake by human cells, indicating that it does recognize or interfere with a GBS-binding site.

CR3-mediated phagocytosis of GBS in such cultures can possibly occur via a C3bi ligand following opsonization of GBS by endogenously secreted complement components from the macrophages (17). Direct recognition of GBS by CR3 without a requirement for C3-mediated opsonization, however, is indicated since phagocytosis of GBS was not significantly inhibited by anti-C3 or salicylhydroxamate. Since endogenous complement synthesis is not a factor in neutrophil studies, anti-Mac-1 inhibition of human neutrophil phagocytosis in serum-free conditions supports the complement independence of Mac-1-mediated uptake of GBS. Studies here with human phagocytes indicate that CR1 is also not involved since anti-CR1 does not inhibit nonopsonic uptake of GBS. CR1 has been shown to contribute to opsonophagocytosis of GBS, and combined anti-CR1 and anti-CR3 are quite inhibitory to GBS uptake and killing in the presence of human serum (41). The role of p150,95, also known as CR4 (28), also implicated in mediating phagocytic events, was not directly evaluated here.

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 function of CR3 in phagocytosis (21). These studies do not clearly differentiate these possibilities; however, general nonopsonic ingestion of latex or *Staphylococcus aureus* (data not shown) is not blocked by anti-Mac-1. Anti-Mac-1 has been shown to inhibit both EC3bi binding (the C3bi or RGD site) and a beta-glucan-binding site on CR3 (35). Studies by Ross et al. (35) in human granulocytes also suggest that particle (zymosan) binding to CR3 can occur at both C3-dependent and C3-independent sites on its distinct alpha chain. Wright et al. (46) have also described two classes of ligands for CR3. C3bi, fibrinogen, and *Leishmania* gp63 possess the RGD (Arg-Gly-Asp) site or related site (38, 43, 46) recognized by the CR3 alpha chain, whereas lipopolysaccharides are recognized by a separate site on CR3 (46). The *Leishmania* lipophosphoglycan and lipopolysaccharide are recognized by similar sites (43). Our studies with soluble anti-CD18 effects on GBS uptake by human cells but not with mouse cells resemble results of others showing anti-CD18 inhibition of uptake or binding of organisms such as *S. epidermidis* (37) and particle-bound lipophosphoglycan (43). Opsonophagocytosis studies of GBS by Smith et al. (41) compared inhibitory effects of multiple anti-CD11b antibodies which recognize different sites. Their results indicate a major role for the lectin site on CR3 in opsonophagocytosis and killing of GBS.

The inability to completely block phagocytosis with anti-Mac-1 indicates that additional opsonin-independent recognition and binding sites contribute to phagocytosis of GBS. The reported presence of functionally distinct subsets of CR3 on macrophages not distinguished by anti-Mac-1 (7) might also explain any lack of correlation of phagocytosis with absolute levels of Mac-1. Graham et al. (21) suggest that two distinct populations of CD11b/CD18 molecules exist on human phagocytes. One population is hypothesized to mediate C3bi rosetting, while another is involved in the general phagocytic function of the cell (mediated via several different receptors), perhaps by providing a necessary link between the adherent target and the phagocytic cell's cytoskeleton. Interaction of GBS with a mobile Mac-1 site within the cell membrane was indicated by the down-modulation of CR3 (reduced EC3bi rosettes) on macrophages adherent to GBS-coated culture plates. In addition, macrophages adherent to anti-Mac-1-coated plates exhibited specific down-modulation of Mac-1 and a reduction in GBS phagocytosis. Our data demonstrate a GBS interaction with mobile Mac-1 but do not exclude a role for the nondiffusible epitope in phagocytosis. Since both the quantity and functional quality (adhesion) of leukocyte adhesion molecules change in response to cytokines and to adhesive substrates (1, 3), it is quite possible that Mac-1 levels versus function will not directly correlate between differentially activated macrophage populations.

Conflicting reports exist as to whether the C3bi site or another site on CR3 actually triggers cell activation versus phagocytosis (33, 35). Cain et al. (10) report that for opsonized zymosan, the C3bi site mediates binding, whereas ingestion and superoxide burst are triggered via a separate beta-glucan-binding site on CR3. Resident peritoneal macrophages reportedly bind targets via CR3 (EC3bi) but do not ingest them (48). Since GBS are ingested by resident peritoneal macrophages, this indicates that GBS are interacting with a site, other than the C3bi site, which triggers ingestion.

Insufficient comparisons of phagocytic indices for GBS strains and serotypes were conducted to determine whether the bacterial ligand for CR3 is a uniform characteristic; however, Smith et al. (41) report a differential inhibition of type Ia versus type III GBS opsonophagocytosis by antibodies to CR3. The chemical nature of the GBS ligand for CR3 is unknown, but GBS polysaccharides may be recognized by the reported lectinlike activity of CR3 (10, 35, 41). The use of heat-killed GBS in this study may introduce artificial phagocytic responses; however, phagocyte bactericidal studies (data not shown) with living GBS show dependence on similar nonopsonic mechanisms. Smith et al. (41) found a similar CR3 dependence for opsonophagocytosis of living GBS by human neutrophils. Inhibition or activation of phagocyte functions following binding of CR3 by the GBS ligand are likely to play a role in both infectious outcome and GBS-induced inflammation (42).

GBS virulence correlates with elevated secretion of type antigens (13, 25) and resistance to phagocytosis in the absence of specific opsonins (2, 15, 44). GBS virulence is partially related to poor opsonophagocytosis via the alternate complement pathway (16); therefore, relative nonopsonic uptake may be a virulence determinant of GBS strains and recognition via adhesion molecules may be an important early nonspecific defense. Since immunity to GBS rests primarily on effective phagocytosis promoted by polysaccharide type-specific antibodies and complement (4, 16), the significance of opsonin-independent CR3-mediated uptake must be determined by virulence studies of GBS versus CR3 interactions. The interaction of GBS with CR3 may be a key

triggering event in both opsonin-dependent and opsonin-independent phagocytosis.

The basis for the susceptibility of neonates versus the resistance of adults to GBS infection still remains an area for speculation and investigation. The predominant hypotheses for neonatal susceptibility to GBS are based on evidence of (i) decreased complement levels and function in neonates versus adults (reviewed in reference 14) or (ii) impaired phagocyte migration and impaired phagocytosis or killing in neonates versus adults (6, 22, 26, 30, 39). Fleit (19) reported that similar percentages of adult versus cord blood polymorphonuclear phagocytes express FcRγIII, CR1, and CR3. Fleit (19) also indicated that CR1 and CR3 expression increased in cord cells treated with the chemotactic peptide FMLP. In contrast, Bruce et al. (8) and Anderson et al. (1) report impaired induction, compared with adults, of surface CR3 (Mac-1) in neonatal granulocytes stimulated by formyl methionyl leucyl phenylalanine (FMLP) and other chemotactic factors. The decreased up-regulation in neonatal cells of CR3, required for phagocyte adherence and migration, was postulated to play a role in impaired inflammatory response in neonates. Smith et al. (41) found that combined antibody blockade of both CR1 and the lectinlike site of CR3 significantly reduced killing of GBS by neonatal phagocytes. Whereas phagocytosis of many bacteria is similar in neonatal versus adult phagocytes (26), reduced levels or function of CR3 in neonatal versus adult phagocytes might explain the differential susceptibility of neonates to bacteria whose uptake is CR3 dependent.

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