INFECTION AND IMMUNITY, June 1996, p. 2206–2215 Vol. 64, No. 6
0019-9567/96/$04.00+0

Leishmania major-Human Macrophage Interactions: Cooperation between Mac-1 (CD11b/CD18) and Complement Receptor Type 1 (CD35) in Promastigote Adhesion

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Received 27 December 1995/Returned for modification 7 February 1996/Accepted 1 April 1996

It has been suggested that the developmental maturation of *Leishmania major* promastigotes can affect their interaction with human complement receptors. To study this, we measured the adhesion of metacyclic and logarithmic-phase *L. major* promastigotes to complement receptors expressed on primary macrophages, to recombinant receptors expressed on transfected cells, or to purified complement receptors in a cell-free system. We demonstrate that complement-opsonized promastigotes can bind to both Mac-1 and complement receptor type 1 (CR1) and that the transition of promastigotes from the noninfectious logarithmic phase of growth to the infectious metacyclic stage does not affect this interaction. Furthermore, we show that Mac-1 and CR1 can cooperate to mediate the efficient adhesion of complement-opsonized metacyclic promastigotes to cells expressing both receptors. On human monocyte-derived macrophages, Mac-1 appears to make a quantitatively greater contribution to this adhesion than does CR1, since blocking macrophage Mac-1 diminishes metacyclic promastigote adhesion to a greater extent than does blocking CR1. In addition, bovine monocytes lacking Mac-1 exhibit a dramatic decrease in complement-dependent promastigote adhesion, relative to normal monocytes. The predominance of Mac-1 in these interactions is due, at least in part, to the factor I cofactor activity of CR1, which facilitates the conversion of C3b to iC3b. The stable adhesion of complement-opsonized metacyclic promastigotes to Mac-1 is a prerequisite for phagocytosis by human monocyte-derived macrophages. Blocking Mac-1 on macrophages abrogates the majority of the complement-dependent phagocytosis of promastigotes, whereas blocking CR1 has no detectable effect on phagocytosis. In addition, bovine monocytes lacking Mac-1 exhibit a dramatic reduction in promastigote phagocytosis relative to normal bovine monocytes. We conclude, therefore, that the two complement receptors, Mac-1 and CR1, can cooperate to mediate the initial complement-dependent adhesion of metacyclic promastigotes to human monocyte-derived macrophages and that Mac-1 is the predominant complement receptor responsible for the phagocytosis of complement-opsonized metacyclic promastigotes.

Protozoa of the genus *Leishmania* are dimorphic obligate intracellular protozoan parasites that reside within mononuclear phagocytes in the mammalian host. The promastigote form of *Leishmania* spp. is delivered to the mammalian host by an infected sandfly. Promastigotes bind to specific receptors on macrophages and are internalized by receptor-mediated phagocytosis (30). This initial promastigote-macrophage interaction is crucial for the establishment of infection in the host. We (26, 27, 29, 31, 32) and others (4, 7, 13, 19, 43, 45–47, 51) have provided evidence for the involvement of a variety of ligands and receptors in the adhesion of promastigotes to macrophages. The direct binding of parasite-derived ligands to receptors on macrophages (4, 13, 19, 29, 32, 43, 45–47) and the indirect binding of promastigotes to macrophages via parasite-associated, host-derived serum opsonins (7, 26, 27, 31, 51) have both been shown to contribute to promastigote adhesion.

Promastigotes fix complement from nonimmune serum (26). Opsonization with the third component of complement, C3, results in a striking enhancement of the binding and phagocytosis of *Leishmania major* promastigotes by macrophages (26, 31). Fixation of C3 by *L. major* promastigotes also dramatically enhances their survival within macrophages (28, 37). These C3-dependent aspects of parasite adhesion, internalization, and intracellular survival result from the interaction of the C3-opsonized promastigotes with complement receptors expressed on macrophages (4, 27, 28).

Human macrophages express three different types of receptors that could potentially mediate the C3-dependent adhesion of *L. major* promastigotes. The leukocyte integrin Mac-1 (CD11b/CD18; $\alpha_M\beta_2$), also known as complement receptor type 3 (CR3), is a heterodimeric receptor that binds to a variety of cell surface and soluble ligands (1, 2, 10, 42, 48, 50), including iC3b, a proteolytically cleaved form of C3b (3, 31, 49). Complement receptor type 1 (CR1; CD35), a member of the regulator of complement activation family, is a monomeric receptor that preferentially binds to C3b and C4b (20). In addition to binding to C3b and C4b, CR1 possesses decay-accelerating factor activity and serves as a cofactor for the factor I-mediated cleavage of C3b and C4b (11, 16, 20, 23). Factor I is found in serum at a concentration of 30 to 40 μg/ml (41). In the presence of an appropriate cofactor, factor I mediates the proteolytic cleavage of C3b to iC3b and subsequently to C3c and

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C3dg (14, 36). The ligands for another leukocyte integrin, p150,95 (CD11c/CD18; $\alpha_x\beta_2$), are less well characterized than those for either Mac-1 or CR1 but appear to consist of both cell surface and soluble molecules, including iC3b (24, 33).

The relative contribution of the different macrophage complement receptors to *L. major* promastigote adhesion remains controversial. Previous work has demonstrated that, in the presence of opsonic complement, *L. major* promastigotes bind to Mac-1 expressed on macrophages (27, 31). Furthermore, the majority of the complement-dependent binding of *L. major* promastigotes to human monocyte-derived macrophages could be inhibited by monoclonal antibodies specific for Mac-1 (31). Other investigators, however, have provided evidence that CR1 is the predominant mediator of the complement-dependent binding of *L. major* promastigotes to human monocyte-derived macrophages (7). These apparently contradictory observations could have resulted from differences in the strains or the developmental maturation of the promastigotes used in these studies (7, 31). In the sandfly midgut, *Leishmania* promastigotes undergo a process of programmed differentiation from a relatively noninfective stage to a highly infective metacyclic stage (22, 38, 39). This process is also observed in axenic culture, in which relatively noninfective promastigotes from logarithmic-phase cultures develop into more infective organisms when the cultures reach stationary phase (6, 38, 39). Highly infective metacyclic promastigotes can be isolated from stationary-phase cultures on the basis of their failure to be agglutinated by the lectin, peanut agglutinin (6, 38).

In order to clarify the respective roles of Mac-1 and CR1 in the complement-dependent adhesion of different developmental forms of *L. major* promastigotes, we examined the binding of metacyclic and logarithmic-phase promastigotes to human Mac-1 and CR1. We demonstrate that, in the presence of opsonic complement, both metacyclic and logarithmic-phase promastigotes can bind to either Mac-1 or CR1. To determine the relative importance of these receptors to promastigote adhesion, we studied the binding of metacyclic *L. major* promastigotes to transfected cells coexpressing Mac-1 and CR1 and to human monocyte-derived macrophages. On both types of cells, Mac-1 and CR1 can cooperate to mediate the initial complement-dependent adhesion of metacyclic promastigotes, but on monocyte-derived macrophages, Mac-1 appears to play a quantitatively greater role than does CR1. Mac-1 is also the predominant complement receptor mediating the internalization of complement-opsonized metacyclic *L. major* promastigotes by monocyte-derived macrophages.

MATERIALS AND METHODS

L. major. The Friedlin strain of *L. major*, clone V1 (MHOM/IL/80/Friedlin), was kindly provided by David Sacks (National Institutes of Health, Bethesda, Md.) (6, 7). The promastigotes were cultured in Schneider's drosophila medium (GIBCO BRL, Grand Island, N.Y.) supplemented with 20% fetal calf serum (FCS) (HyClone, Logan, Utah), glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Stationary-phase promastigotes were obtained from 5- to 7-day-old cultures. Metacyclic promastigotes were selected from stationary-phase cultures by agglutination with peanut agglutinin (Vector Laboratories, Burlingame, Calif.) as described by Da Silva and Sacks (6). Logarithmic-phase promastigotes were maintained by daily passage. Stationary-phase cultures were radiolabeled by the addition of 5 μCi/ml of [6-^{3}H]uracil (20 to 30 Ci/mmol) (Du Pont NEN, Boston, Mass.) or [5,6-^{3}H]uracil (40 to 60 Ci/mmol) (Amersham, Arlington Heights, Ill.), at the initiation of culture. Logarithmic-phase cultures were radiolabeled overnight with 5 μCi/ml of [^{3}H]uracil.

Antibodies. Monoclonal antibody (MAb) CBRM1/29 (mouse immunoglobulin G1 [IgG1], anti-human CD11b) (8) was purified from ascitic fluid. The hybridoma was provided by Timothy Springer (The Center for Blood Research, Harvard Medical School, Boston, Mass.). This MAb reacts with Mac-1 but not with CR1. The 3D9 MAb (mouse IgG1, anti-human CR1) (35) was provided by John O'Shea (National Institutes of Health). It was diluted from ascitic fluid or used as IgG that had been purified from ascitic fluid. This MAb reacts with CR1 but not with Mac-1. Purified LM2/1 MAb (mouse IgG1, anti-human CD11b) (25) was provided by Timothy Springer. The WIC 79.3 MAb (mouse IgG1, anti-*L. major* lipophosphoglycan) (13) was diluted from ascitic fluid. The hybridoma was provided by Emanuela Handman (Walter and Eliza Hall Institute, Melbourne, Australia). MOPC 21 (mouse IgG1, myeloma protein) was purchased from Sigma (St. Louis, Mo.) and ICN (Costa Mesa, Calif.). Fluorescein isothiocyanate (FITC)-conjugated MAb 44 (mouse IgG1, anti-human CD11b), FITC-conjugated MOPC 21, and R-phycoerythrin-conjugated goat anti-mouse IgG (heavy and light chains) were purchased from Sigma. FITC-conjugated goat anti-mouse IgG (heavy and light chains) was purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa.).

Chinese hamster ovary (CHO) cells transfected with human complement receptors. CHO cells stably expressing wild-type human Mac-1 (CHO–Mac-1.3C5) or a third immunoglobulin-like-domain-deleted form of human ICAM-1 (CHO-F185.1) have been described previously (8, 15). These cell lines were maintained in α-minimal essential medium (GIBCO BRL) supplemented with 10% dialyzed FCS (HyClone), 0.1 μM methotrexate (Lederle Laboratories, Pearl River, N.Y.), 16 μM thymidine, 2 mM glutamine, 100-U/ml penicillin G, and 100-μg/ml streptomycin. To generate CR1 transfectants and Mac-1–CR1 cotransfectants, 10^7 CHO-F185.1 or CHO–Mac-1.3C5 cells, respectively, were cotransfected with 20 μg of paABCD and 1 μg of pRSVneo. Plasmid paABCD was provided by Lloyd Klickstein (The Center for Blood Research). It contains the complete cDNA of the F allotype of human CR1 cloned into the Ap^{r}M8 expression vector (17, 20). Plasmid pRSVneo (12) allowed for the selection of stable transfectants in medium supplemented with G418 sulfate (GIBCO BRL) (500 μg/ml for CR1 transfectants and 800 μg/ml for Mac-1–CR1 cotransfectants). To obtain linear DNA for transfections, paABCD and pRSVneo were digested with *Sfi*I and *Bam*HI, respectively. Transfections were performed by electroporation using 0.4-cm-diameter cuvettes and a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.) set at 0.38 kV and 960 μF. G418-resistant cells were selected, cloned by limiting dilution, and screened for cell surface CR1 expression by flow cytometry. CHO-CR1.1D9 and CHO–Mac-1/CR1.2B12 are clonal cell lines that stably express CR1 or both Mac-1 and CR1, respectively.

For use in *Leishmania*-binding assays, transfected CHO cells (10^5) were allowed to adhere to the plastic wells of 24-well plates for 6 to 10 h. The cells were then washed free of serum and used in the assays.

Human monocyte-derived macrophages. Human peripheral blood was obtained by venipuncture from healthy adult donors. Peripheral blood mononuclear cells were isolated by centrifugation through a Ficoll-Isopaque density gradient (Accurate, Westbury, N.Y.). To obtain monocyte-derived macrophages, the mononuclear cells were added to Teflon beakers (Savilex, Minnetonka, Minn.) and cultured in RPMI-1640 (Mediatech, Herndon, Va.) supplemented with 5% autologous serum, glutamine (2 mM), penicillin G (100 U/ml), streptomycin (100 μg/ml), and recombinant human macrophage colony-stimulating factor (30 ng/ml) (Immunex, Seattle, Wash.) for 5 days at 37°C in 5% CO_2. These cells were then allowed to adhere to 13-mm-diameter glass coverslips in 24-well plates in the presence of 5% autologous serum for 2 h at 37°C. Nonadherent cells were removed by washing, and the monolayers were used the following day for binding assays. The cells were washed free of serum prior to use in *Leishmania*-binding and phagocytosis assays.

Bovine monocytes. Bovine monocytes were isolated from the peripheral blood of normal Holstein calves and calves with bovine leukocyte adhesion deficiency disease (BLAD) (40). The calves were raised at the National Animal Disease Center-USDA in Ames, Iowa. Leukocytes from animals with BLAD have previously been shown by flow cytometry (40) and lectin immunoblot analysis (18) to be devoid of Mac-1. Mononuclear cells were obtained from buffy coat leukocytes by separation over a Percoll density gradient (Pharmacia, Piscataway, N.J.). Mononuclear cells were resuspended in RPMI-1640 and were allowed to adhere to 13-mm-diameter glass coverslips in 24-well plates in the presence of 5% fetal bovine serum for 1 h at 37°C. Nonadherent cells were removed by washing, and the monolayers were used immediately for *Leishmania* adhesion and phagocytosis assays. These assays were performed as described for human monocyte-derived macrophages, except that 4% normal bovine serum was used as a source of complement.

Flow cytometry. Transfected CHO cells (10^6) were incubated with MAbs for 45 min at 4°C. The cells were washed three times with cold wash buffer (Hanks' balanced salt solution [HBSS] [GIBCO BRL] with 1% bovine serum albumin [BSA] and 0.05% sodium azide) and incubated with FITC-conjugated goat anti-mouse IgG for 45 min at 4°C. After being washed, the cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS). For two-color immunofluorescent staining, R-phycoerythrin-conjugated goat anti-mouse IgG was substituted for FITC-conjugated goat anti-mouse IgG, and an additional 45-min incubation at 4°C was carried out with FITC-conjugated 44 or FITC-conjugated MOPC-21 prior to paraformaldehyde fixation. The cells were analyzed on an Epics Elite flow cytometer (Coulter Diagnostics, Hialeah, Fla.).

Complement receptor-coated substrates. Affinity-purified human Mac-1 (a gift from Timothy Springer) was adsorbed to 96-well plates as previously described (31). Soluble CR1 was provided by Giora Feuerstein (SmithKline Beecham Pharmaceuticals, King of Prussia, Pa.). A total of 50 μl of sCR1 at a concentration of 200 μg/ml in HBSS was added to wells of non-tissue culture-treated 96-well plates for 90 min at room temperature. After being washed, the plates were blocked with 1% BSA in HBSS for 2 h at 37°C and stored at 4°C until used.

The density of receptors on the Mac-1–coated plates was previously estimated to be comparable to that expressed on activated neutrophils (8).

***Leishmania*-binding and phagocytosis assays.** Washed radiolabeled promastigotes were added to 24-well plates containing either human monocyte-derived macrophages or transfected CHO cells. All binding assays were performed in triplicate in a buffer consisting of equal parts of medium 199 and Dulbecco's modified Eagle medium (Mediatech) supplemented with 1% BSA and 12.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). In assays performed in the presence of serum, a final concentration of 4% human serum from an individual deficient in the eighth component of complement, C8D serum, was added to each well. The use of C8D serum obviates any problems associated with complement-mediated lysis of parasites, particularly the complement-sensitive logarithmic-phase promastigotes (5, 6). After a 45-min incubation at 35°C, unbound promastigotes were removed by thorough washing. The monolayers and bound promastigotes were solubilized with 0.5% Triton X-100, and the amount of radiolabel associated with these lysates was determined by an LS6500 liquid scintillation counter (Beckman, Palo Alto, Calif.). The number of promastigotes bound per well was deduced by using the amount of radiolabel associated with a known number of promastigotes as a conversion factor, as previously described (26). In assays involving antibody inhibition of promastigote binding or internalization, the monolayers were preincubated with the antibodies at a final concentration of 20 μg/ml for 30 min at 35°C prior to the addition of the parasites, and the antibodies remained in the wells for the duration of the assay. In some experiments, purified human factor I (Quidel) was added to wells at a final concentration of 10 μg/ml. In these experiments, instead of opsonizing the parasites by the direct addition of C8D serum to the wells, the promastigotes (5×10^7 cells per ml) were opsonized with 10% C8D serum for 15 min at 35°C, washed thoroughly, and then added to the wells.

Binding assays involving complement receptor-coated wells were performed in a similar manner, except that in all experiments the promastigotes were opsonized with 10% C8D, as described above, prior to their addition to the wells. In addition, bound parasites were solubilized with 0.5% Triton X-100 and 0.1 N NaOH. The lysates were then neutralized with 0.1 N HCl prior to liquid scintillation counting.

Assays to measure internalization of promastigotes by human monocyte-derived macrophages were performed in a manner similar to the binding assays, except that after a 1-h incubation at 35°C in the presence of C8D serum, unbound promastigotes were removed by thorough washing and parallel monolayers were differentially fixed with either 100% methanol at 4°C or 1% paraformaldehyde in PBS at room temperature. Parasites were visualized by immunofluorescence microscopy. The monolayers were washed with PBS containing 5% FCS and incubated for 45 min at 4°C with a 1:100 dilution of WIC 79.3 ascitic fluid containing a MAb to *L. major* lipophosphoglycan. After being washed with PBS containing 5% FCS, the monolayers were incubated with FITC-conjugated goat anti-mouse IgG for 45 min at 4°C. After further washing, the coverslips were examined by fluorescence microscopy, counting at least 200 macrophages per coverslip. Methanol fixation permeabilizes the macrophage cell membrane and permits the immunofluorescent staining of extracellular and intracellular leishmaniae, whereas paraformaldehyde fixation only allows the immunofluorescent staining of extracellular leishmania (34). The number of parasites internalized per macrophage was determined by subtracting the number of promastigotes per macrophage in the paraformaldehyde-fixed monolayer from the number of organisms in the methanol-fixed monolayer.

Preparation of erythrocytes coated with C3b and iC3b. The preparation of erythrocytes coated with C3b or iC3b has previously been described in detail (44). Briefly, C3b was prepared from purified C3 (Quidel, San Diego, Calif.) by cleavage with trypsin (36). C3b was coupled to washed sheep erythrocytes (Lampire, Pipersville, Pa.) by a modification of the method described by Lambris and colleagues (21). Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce, Rockford, Ill.), at a final concentration of 0.5 mM, was incubated with sheep erythrocytes (10^9 cells per ml) in HBSS for 60 min at room temperature with continuous rotation. The thiol-activated sheep erythrocytes were washed four times with HBSS, resuspended in C3b (300 μg/ml) to a concentration of 5×10^9 cells per ml, and incubated at room temperature with continuous rotation for 60 min. The resulting C3b-coated erythrocytes (EC3b) were washed three times with HBSS and resuspended to a concentration of 2×10^8 cells per ml. The EC3b were stored at 4°C until use. Erythrocytes coated with iC3b (EC3bi) were generated by incubating EC3b (2×10^8 cells per ml) with 10% heat-inactivated serum for 20 min at 37°C. The EC3bi were washed three times with HBSS, resuspended to a concentration of 2×10^8 cells per ml, and used immediately. Heat-inactivated serum was prepared by incubating normal human serum for 35 min at 56°C.

EC3b and EC3bi binding assays. Monolayers of human monocyte-derived macrophages adhered to 13-mm-diameter glass coverslips were preincubated either with buffer alone or with MAb CBRM1/29 (anti-Mac-1) or MAb 3D9 (anti-CR1) at a final concentration of 20 μg/ml for 15 min at 37°C. EC3b or EC3bi were then added to monocyte-derived macrophages at a ratio of 40:1. The monolayers were incubated with the erythrocytes for 35 min at 37°C. After being washed to remove unbound erythrocytes, the monolayers were fixed with 2.5% glutaraldehyde and stained with Geimsa (Sigma). The number of erythrocytes bound to the monolayers was quantitated by light microscopy by counting at least 200 cells per coverslip.

Statistical analysis. The statistical significance of the data was determined by Student's *t* test. A *P* value of less than 0.05 was taken to be significant.

RESULTS

The binding of metacyclic, stationary-phase, and logarithmic-phase *L. major* promastigotes to CHO cells expressing human Mac-1 or CR1. To examine the adhesion of different developmental stages of *L. major* promastigotes to cell surface Mac-1 or CR1 in the absence of other macrophage receptors, we used CHO cells that had been stably transfected with expression vectors containing the cDNAs for human Mac-1 or CR1. As demonstrated by flow cytometry with MAbs specific for the respective complement receptors, CHO–Mac-1.3C5 cells express high levels of cell surface human Mac-1 (Fig. 1A), and CHO-CR1.1D9 cells express high levels of cell surface human CR1 (Fig. 1B). CHO-F185.1 cells, which were transfected with a third immunoglobulin-like-domain-deleted form of human ICAM-1, express neither Mac-1 nor CR1 (Fig. 1C) and were used as controls.

Radiolabeled metacyclic, stationary-phase, or logarithmic-phase *L. major* promastigotes were added to monolayers of transfected CHO cells in the presence or absence of 4% C8D serum, as a source of opsonic complement. In the presence of serum, promastigotes from all phases of growth bound avidly to Mac-1- or CR1-transfected CHO cells (Fig. 2). This binding was dependent on the presence of fresh serum, because in the absence of serum, the amount of binding was negligible and not significantly different from the amount of binding to control-transfected CHO cells (data not shown). The logarithmic-phase promastigotes bound to Mac-1- or CR1-transfected CHO cells better than did metacyclic or stationary-phase promastigotes (Fig. 2). The receptor specificity of the serum-dependent binding to Mac-1- or CR1-transfected CHO cells was confirmed by inhibiting this binding with MAbs specific for Mac-1 (CBRM1/29) or CR1 (3D9), respectively (Fig. 2A and B). This binding was not inhibited by an isotype-matched control antibody, MOPC 21 (Fig. 2A and B). Thus, in the presence of opsonic complement, metacyclic, stationary-phase, and logarithmic-phase *L. major* promastigotes can bind to either cell surface Mac-1 or CR1.

The binding of metacyclic and logarithmic-phase *L. major* promastigotes to human complement receptor-coated plates. To confirm the results obtained with transfected CHO cells, we added radiolabeled metacyclic and logarithmic-phase *L. major* promastigotes to purified human Mac-1 or human sCR1 adsorbed to plastic plates. Serum-opsonized metacyclic and logarithmic-phase promastigotes bound avidly to Mac-1- and sCR1-coated wells (Fig. 3). This binding was serum dependent, because in the absence of serum opsonization, promastigote adhesion to these receptors was not significantly different from background adhesion to BSA-coated wells (data not shown). Similar to the results observed with transfected CHO cells, logarithmic-phase promastigotes bound to purified Mac-1 or sCR1 better than did metacyclic promastigotes. The receptor specificity of the serum-dependent binding to purified Mac-1 or CR1 was confirmed by the inhibition of binding by MAbs specific for Mac-1 (CBRM1/29) or CR1 (3D9), respectively (Fig. 3). Thus, in the presence of opsonic complement, metacyclic and logarithmic-phase *L. major* promastigotes can bind to either purified Mac-1 or sCR1.

The adhesion of metacyclic *L. major* promastigotes to CHO cells expressing both human Mac-1 and CR1. To determine whether Mac-1 and CR1 cooperate in the adhesion of serum-opsonized *L. major* promastigotes, CHO cells were developed that had been stably transfected with both receptors. The

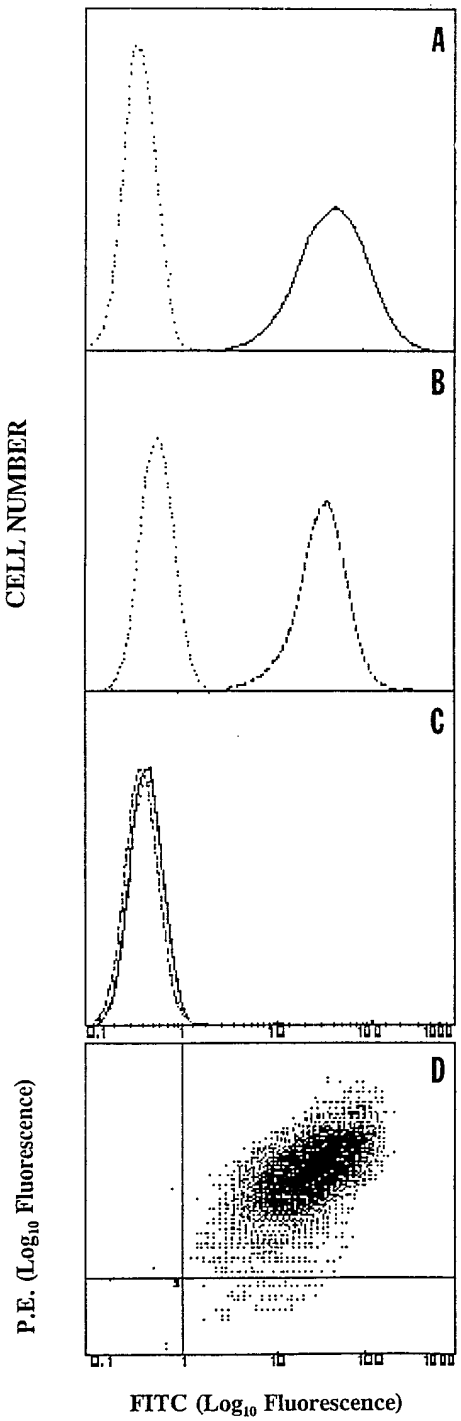

FIG. 1. Cell surface expression of human Mac-1, CR1, or both Mac-1 and CR1 on transfected CHO cells. (A to C) Cells were stained by indirect immunofluorescence with either an isotype-matched control antibody (MOPC 21) (dotted line) or MAbs to Mac-1 (LM2/1) (solid line) or CR1 (3D9) (dashed line) and analyzed by flow cytometry. CHO–Mac-1.3C5 cells (A), CHO-CR1.1D9 cells (B), and CHO-F185.1 cells (C) were stably transfected with human Mac-1, human CR1, and a truncated form of human ICAM-1, respectively. The profiles of CHO–Mac-1.3C5 and CHO-CR1.1D9 cells incubated with 3D9 and LM2/1, respectively, were indistinguishable from those obtained with MOPC 21 (data not shown). (D) CHO–Mac-1/CR1.2B12 cells, which had been stably transfected with both human Mac-1 and CR1, were stained with 3D9 and R-phycoerythrin-conjugated goat anti-mouse IgG and then with FITC-conjugated 44, an MAb to Mac-1. These cells were analyzed by two-color flow cytometry (*x* axis, Mac-1 expression; *y* axis, CR1 expression). Cells stained with control antibodies were used to set the gates.

CHO–Mac-1/CR1.2B12 cells express high levels of both human Mac-1 and CR1 on their cell surface, as demonstrated by flow cytometry with MAbs specific for the respective complement receptors (Fig. 1D). Radiolabeled metacyclic *L. major* promastigotes were added to monolayers of CHO–Mac-1/CR1 cells in the presence or absence of 4% C8D serum. In the presence of serum, metacyclic promastigotes bound avidly to CHO–Mac-1/CR1 cells ($4.05 \times 10^5 \pm 6.3 \times 10^4$ promastigotes bound per well; mean ± standard error [SE], $n = 5$). This binding was dependent on the presence of serum, because when serum was omitted from the assay, the amount of binding did not differ from that observed with control-transfected CHO cells (not shown). To determine the relative contribution of Mac-1 and CR1 to the serum-dependent adhesion of metacyclic promastigotes to CHO–Mac-1/CR1 cells, the assays were performed in the presence of MAbs specific for each receptor. A modest inhibition of serum-dependent promastigote adhesion was observed when either of the MAbs was individually added to the monolayers, with only CBRM1/29 causing a significant ($P < 0.05$) reduction in binding, relative to monolayers treated with an irrelevant antibody, MOPC 21 (Fig. 4). Treatment of CHO–Mac-1/CR1 cells with both MAbs, however, resulted in a dramatic inhibition of serum-dependent promastigote adhesion, reducing binding by 75%, relative to untreated controls (Fig. 4). These results indicate that Mac-1 and CR1 expressed on transfected CHO cells can cooperate in mediating the serum-dependent binding of metacyclic *L. major* promastigotes.

The adhesion of metacyclic *L. major* promastigotes to human monocyte-derived macrophages. We next examined complement receptor cooperativity on human monocyte-derived macrophages. These macrophages express functional CR1 and Mac-1 and readily form rosettes with sheep erythrocytes coated with C3b and iC3b, respectively (Fig. 5). The addition of MAb to CR1 (3D9) inhibited the rosetting of C3b-coated erythrocytes but not that of iC3b-coated erythrocytes. The addition of MAb to Mac-1 (CBRM1/29) inhibited the rosetting of iC3b-coated erythrocytes but not that of C3b-coated erythrocytes (Fig. 5). Radiolabeled metacyclic *L. major* promastigotes were added to human monocyte-derived macrophages in the absence or presence of 4% C8D serum. Consistent with our previously published observations (31), in the absence of serum, promastigotes bound poorly to human monocyte-derived macrophages ($3.1 \times 10^4 \pm 3 \times 10^3$ promastigotes per monolayer; mean ± SE, $n = 5$). In the presence of serum, however, metacyclic promastigotes bound avidly to monocyte-derived macrophages ($2.9 \times 10^5 \pm 4.1 \times 10^4$ promastigotes bound per monolayer). To determine the relative contribution of each receptor to this serum-dependent adhesion, MAbs to Mac-1 or CR1 or both were added to these assays. The addition of MAb to Mac-1 inhibited promastigote binding by 49% ($P < 0.05$), whereas the addition of a blocking MAb specific for CR1 failed to significantly inhibit binding (Fig. 6). Similar to our observations with the cotransfected CHO cells, the simultaneous addition of both blocking MAbs further diminished promastigote adhesion, reducing the number of attached promastigotes by 68%, relative to control levels (Fig. 6). These results suggest that Mac-1 and CR1 cooperate in mediating the complement-dependent adhesion of metacyclic *L. major* promastigotes to monocyte-derived macrophages. Mac-1, however, appears to play a quantitatively greater role than CR1 in mediating the serum-dependent adhesion of promastigotes to macrophages.

The effect of human factor I on the adhesion of metacyclic *L. major* promastigotes to human CR1. Serum contains the complement regulatory protein, factor I. To determine if the factor I cofactor activity of CR1 could influence the comple-

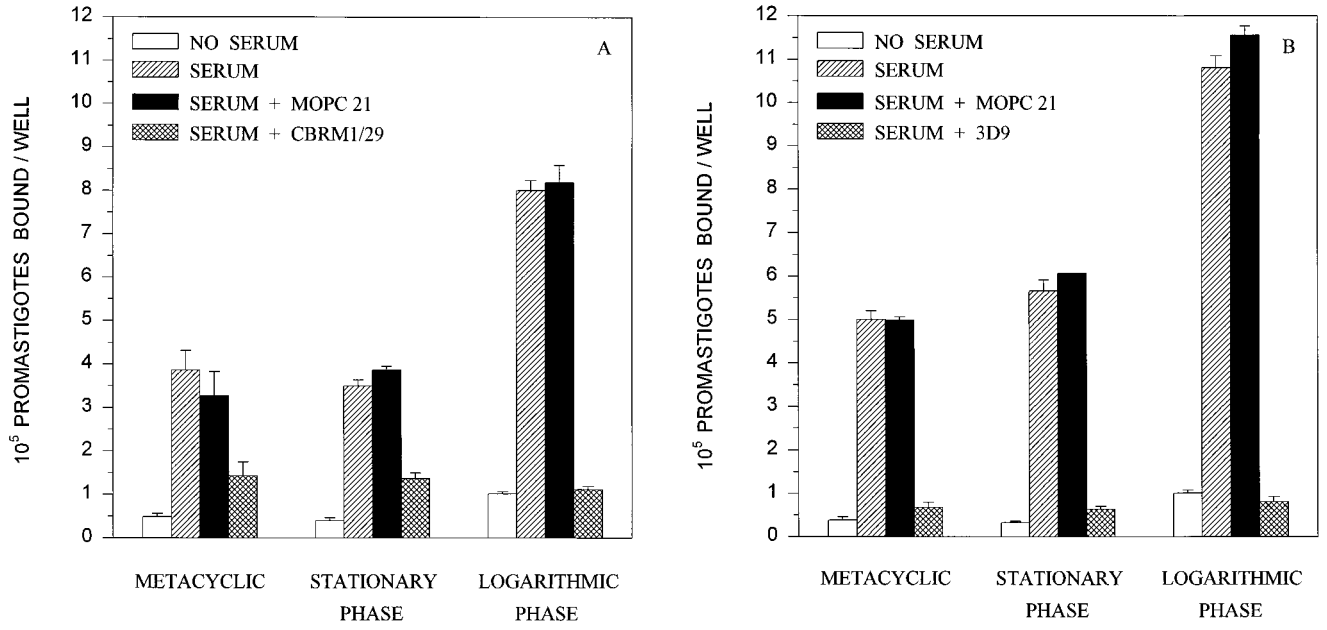

FIG. 2. The binding of metacyclic, stationary-phase, and logarithmic-phase *L. major* promastigotes to transfected CHO cells expressing either human Mac-1 or CR1. A total of 2.5×10^6 radiolabeled promastigotes were added in the presence or absence of 4% C8D serum to monolayers of CHO cells expressing either human Mac-1 (CHO–Mac-1.3C5 cells) (A) or human CR1 (CHO-CR1.1D9 cells) (B). After a 45-min incubation at 35°C, unbound promastigotes were removed by washing, and the amount of radiolabeled promastigotes associated with each monolayer was determined. Some monolayers were preincubated either with MAb to Mac-1 (CBRM1/29) or CR1 (3D9) or with a control antibody (MOPC 21) for 30 min prior to the addition of the promastigotes. Values are the means of triplicate determinations ± SD. This experiment is representative of three independent experiments.

ment-dependent adhesion of metacyclic *L. major* promastigotes to CR1, we added complement-opsonized radiolabeled metacyclic promastigotes to monolayers of transfected CHO cells expressing complement receptors in either the presence or absence of purified human factor I. The addition of factor I caused a dramatic inhibition of promastigote adhesion to CHO-CR1 cells (Fig. 7A). In three independent experiments, the addition of factor I reduced promastigote adhesion by 66% ± 3% (mean ± SE) relative to parallel monolayers to which no factor I was added. In contrast, the addition of factor

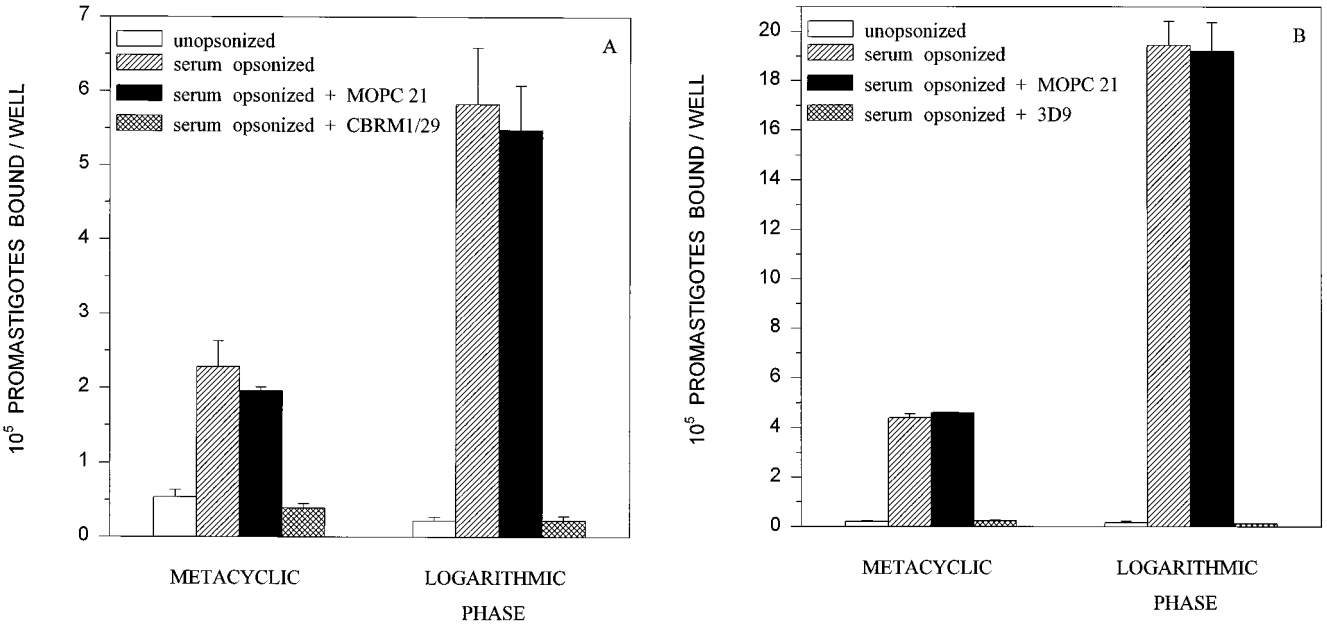

FIG. 3. The binding of metacyclic and logarithmic-phase *L. major* promastigotes to purified Mac-1- or sCR1-coated plates. A total of 2.5×10^6 radiolabeled promastigotes, either unopsonized or opsonized with 10% C8D serum for 15 min, were added to plates coated with affinity-purified human Mac-1 (A) or sCR1 (B). After a 45-min incubation at 35°C, unbound promastigotes were removed by washing, and the amount of radiolabeled promastigotes associated with each well was determined. Some wells were preincubated either with MAb to Mac-1 (CBRM1/29) or CR1 (3D9) or with a control antibody (MOPC 21) for 30 min prior to the addition of the promastigotes. Values are the means of triplicate determinations ± SD. This experiment is representative of three independent experiments.

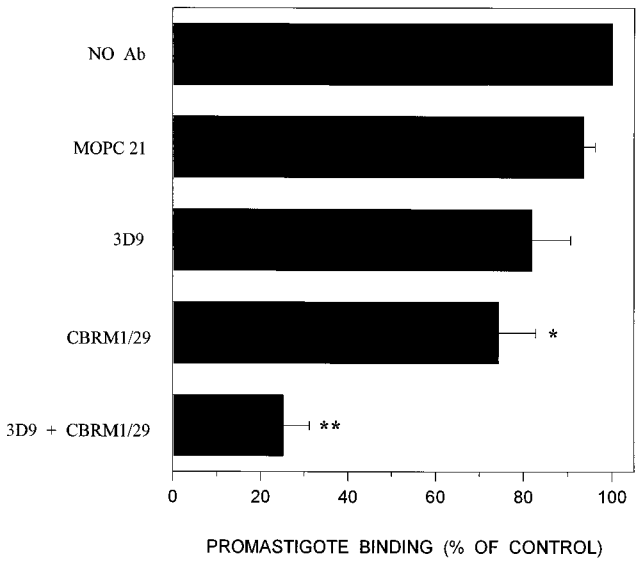

FIG. 4. The effect of MAbs to Mac-1 and CR1 on the serum-dependent binding of metacyclic *L. major* promastigotes to CHO cells expressing both human Mac-1 and CR1. A total of 2.5×10^6 radiolabeled metacyclic promastigotes were added in the presence of 4% C8D serum to monolayers of CHO–Mac-1/CR1.2B12 cells. After a 45-min incubation at 35°C, unbound promastigotes were removed by washing, and the amount of radiolabeled promastigotes associated with each monolayer was determined. Some monolayers were preincubated either with MAb to Mac-1 (CBRM1/29) or CR1 (3D9) or with a control antibody (MOPC 21) for 30 min prior to the addition of the promastigotes. Data are presented as percentages of the control value, which is the number of promastigotes bound to monolayers in the presence of serum but in the absence of antibodies. The amount of promastigote binding in the absence of serum was subtracted from all values. Values are the means of five independent experiments performed in triplicate ± SE. *, significant difference ($P < 0.05$) from monolayers treated with MOPC 21; **, significant difference ($P < 0.01$) from monolayers treated with either 3D9 or CBRM1/29.

I to monolayers of CHO–Mac-1 cells had no effect on promastigote adhesion (Fig. 7A). Promastigotes remained stably associated with Mac-1-transfected cells even after prolonged incubation of monolayers in factor I. A similar observation was made with purified receptors adsorbed to plastic. The addition of factor I decreased promastigote adhesion to wells coated with sCR1 (Fig. 7B) but had no effect on the adhesion of promastigotes to wells coated with Mac-1 (Fig. 7B). In three separate experiments, the addition of factor I reduced promastigote adhesion to sCR1-coated wells by 83% ± 2% (mean ± SE), relative to wells to which no factor I was added. These data suggest that the adhesion of promastigote-associated C3b to CR1 may be transient because of the cofactor activity of CR1. This cofactor activity can facilitate the cleavage of C3b by factor I to a form that is no longer recognized by CR1.

The phagocytosis of metacyclic *L. major* promastigotes by human monocyte-derived macrophages and bovine monocytes. To determine the relative roles of Mac-1 and CR1 in the phagocytosis of complement-opsonized metacyclic *L. major* promastigotes by monocyte-derived macrophages, organisms were added to cell monolayers treated with MAbs to Mac-1 or CR1. The extent of promastigote internalization by macrophages was determined. Monolayers were differentially fixed to distinguish internalized versus peripherally attached promastigotes when stained by immunofluorescence. In control monolayers, the phagocytosis of metacyclic promastigotes by macrophages was a rapid process, and by 1 h, 85% ± 7% of the organisms associated with the monolayer were inside macrophages (mean ± standard deviation [SD], $n = 4$). Incubating the monolayers with MAb to CR1 (3D9) had no effect on promastigote internalization (Fig. 8), indicating that Mac-1 was sufficient to mediate the complement-dependent internalization of metacyclic promastigotes. The addition of MAb to Mac-1 resulted in a significant decrease in the number of promastigotes inside macrophages, relative to untreated monolayers (Fig. 8). The simultaneous addition of both CBRM1/29 and 3D9 to monolayers resulted in no additional inhibition of phagocytosis over that seen with MAb to Mac-1 alone (Fig. 8).

Bovine monocytes isolated from the peripheral blood of calves with BLAD were used to further study the role of Mac-1 in promastigote phagocytosis. Metacyclic promastigotes were added to normal or BLAD monocytes in the presence or absence of 4% bovine serum (as a source of complement), and the extent of promastigote adhesion and phagocytosis was measured after 1 h. With normal bovine monocytes, the presence of serum resulted in an eightfold increase in the number of promastigotes associated with the monolayer, relative to the number observed when the assays were done under serum-free conditions (Fig. 9). The majority (65%) of these organisms were found inside monocytes. In contrast to normal bovine monocytes, BLAD monocytes exhibited less than a twofold increase in the number of parasites associated with them when the assays were performed in serum, and only 36% of these organisms were inside macrophages (Fig. 9). Thus, the addition of complement caused a dramatic increase in promastigote adhesion to normal bovine monocytes expressing Mac-1, and this adhesion rapidly led to internalization. On BLAD monocytes lacking Mac-1, however, the presence of complement caused only a modest increase in promastigote adhesion, and the efficiency of internalization was diminished. Therefore, Mac-1 appears to be the predominant complement receptor

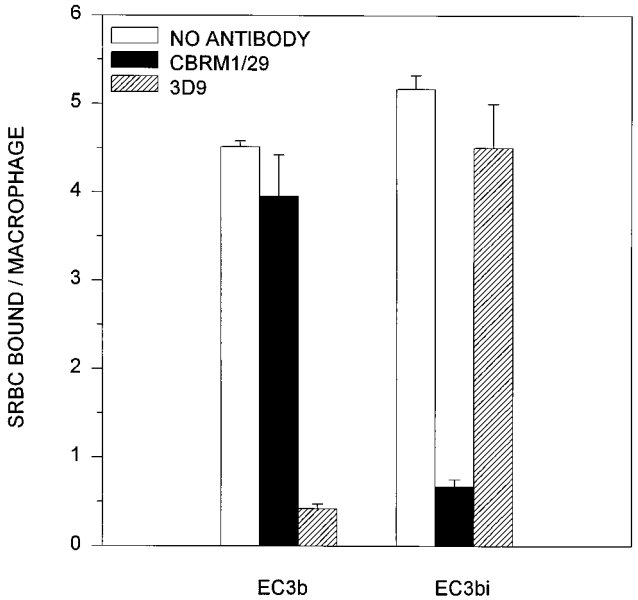

FIG. 5. The binding of erythrocytes (SRBC) coated with C3b or iC3b to human monocyte-derived macrophages. Erythrocytes coated with C3b (EC3b) or iC3b (EC3bi) were added to monolayers of human monocyte-derived macrophages at a ratio of 40:1. Some monolayers were preincubated with MAb to either Mac-1 (CBRM1/29) or CR1 (3D9) for 15 min prior to the addition of erythrocytes. Binding proceeded for 35 min at 37°C and was followed by washing to remove unbound EC3b or EC3bi. Determinations were performed in triplicate and values are means ± SD. This figure is representative of two experiments.

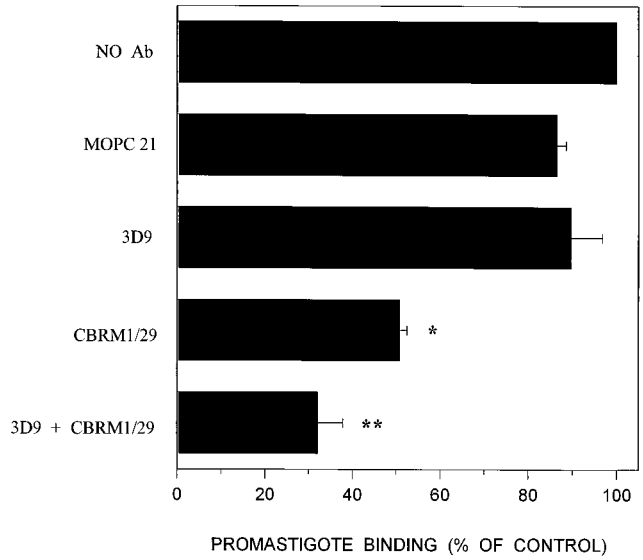

FIG. 6. The effect of MAbs to Mac-1 and CR1 on the serum-dependent binding of metacyclic *L. major* promastigotes to human monocyte-derived macrophages. A total of 2.5×10^6 radiolabeled metacyclic promastigotes were added in the presence of 4% C8D serum to monolayers of monocyte-derived macrophages. After a 45-min incubation at 35°C, unbound promastigotes were removed by washing, and the amount of radiolabeled promastigotes associated with each monolayer was determined. Some monolayers were preincubated either with MAb to Mac-1 (CBRM1/29) or CR1 (3D9) or with control antibody (MOPC 21) for 30 min prior to the addition of the promastigotes. The amount of promastigote binding in the absence of serum was subtracted from all values. Data are presented as percentages of the control value, which is the number of promastigotes bound to monolayers in the presence of serum but in the absence of antibodies. Values are the means of five independent experiments performed in triplicate with macrophages from three different donors ± SE. *, significant difference ($P < 0.01$) from monolayers treated with MOPC 21; **, significant difference ($P < 0.05$) from monolayers treated with CBRM1/29.

mediating the complement-dependent phagocytosis of metacyclic *L. major* promastigotes by mononuclear phagocytes.

DISCUSSION

The interaction of *L. major* promastigotes with complement and complement receptors is a dynamic process that has important ramifications for the establishment of infection in host mononuclear phagocytes. Following opsonization with C3, a striking enhancement in the binding and phagocytosis of *L. major* promastigotes by macrophages (27, 31) and a dramatic enhancement in the intracellular survival of the organisms have been observed (28, 37). A general consensus exists that complement receptor-dependent phagocytosis of *L. major* promastigotes is an important mode of entry for parasites into macrophages. However, the relative contributions of the different macrophage complement receptors to the binding and phagocytosis of *L. major* promastigotes remain controversial. We and others have previously shown that complement-opsonized promastigotes can bind to Mac-1 expressed on macrophages (4, 27, 31). We have also demonstrated that, in the presence of complement, *L. major* promastigotes can bind to affinity-purified Mac-1 and to recombinant Mac-1 expressed on transfected fibroblastoid cells (31). Other investigators, however, have proposed that CR1 is the primary receptor mediating the complement-dependent adhesion of metacyclic promastigotes to human monocyte-derived macrophages (7). In this work, we address the issue of whether the developmental stage of the promastigotes affects their inherent ability to bind to Mac-1 or CR1. We found that both metacyclic and logarithmic-phase *L. major* promastigotes bound efficiently to Mac-1 and to CR1. In the presence of complement, both developmental forms bound to recombinant receptors expressed on fibroblastoid cells and to purified receptors immobilized on plastic. Thus, in these two defined model systems, where no other macrophage receptors can contribute to parasite adhesion, we definitively demonstrate that both Mac-1 and CR1 can

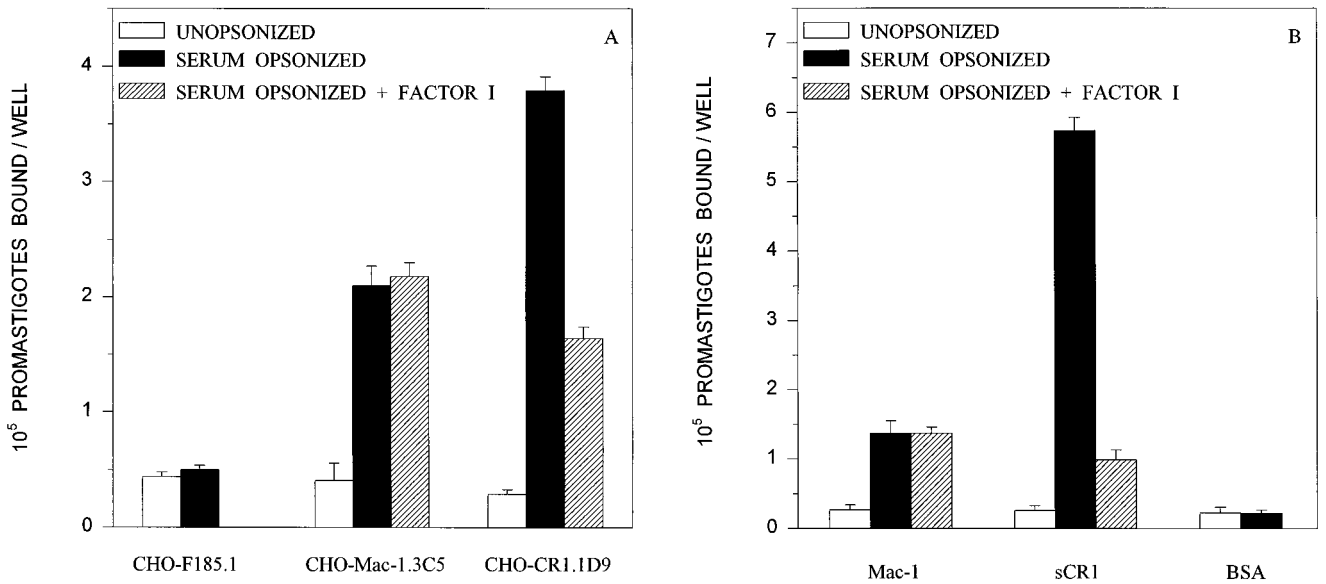

FIG. 7. The effect of human factor I on the serum-dependent binding of metacyclic *L. major* promastigotes to human complement receptors. A total of 2.5×10^6 radiolabeled metacyclic promastigotes were added to monolayers of transfected CHO cells expressing complement receptors (A) or to plates coated with affinity-purified complement receptors (B). After a 45-min incubation at 35°C, unbound promastigotes were removed by washing, and the amount of radiolabeled promastigotes associated with each well was determined. Factor I, at a final concentration of 10 μg/ml, was added to some wells. Values are means of triplicate determinations ± SD, and the experiments shown are each representative of three independent experiments.

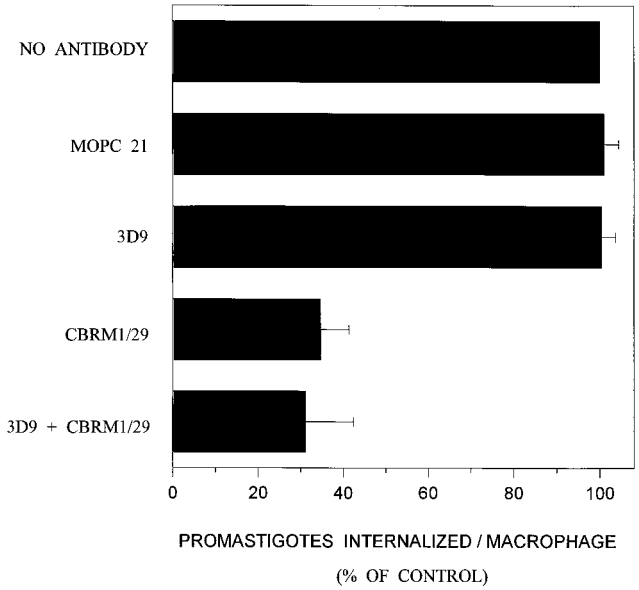

FIG. 8. The effect of MAbs to Mac-1 and CR1 on the serum-dependent phagocytosis of metacyclic *L. major* promastigotes by human monocyte-derived macrophages. A total of 2.5×10^6 metacyclic promastigotes were added in the presence of 4% C8D serum to monolayers of monocyte-derived macrophages. Monolayers were preincubated either with MAb to Mac-1 (CBRM1/29) or CR1 (3D9) or with a control antibody (MOPC 21) for 30 min prior to the addition of the promastigotes. After a 1-h incubation at 35°C, unbound promastigotes were removed by washing, and parallel monolayers were fixed in either methanol or paraformaldehyde. Macrophage-associated *L. major* was stained by indirect immunofluorescence and visualized by fluorescence microscopy. Data are presented as percentages of the control value, which is the number of promastigotes internalized per macrophage in the absence of antibodies. Values are means of four independent experiments with macrophages from four different donors ± SD. The average number of promastigotes internalized per macrophage in the absence of antibodies was 4.19 ± 2.26 (mean ± SD, $n = 4$).

mediate the complement-dependent adhesion of *L. major* promastigotes. The specificity of this adhesion was confirmed by blocking promastigote adhesion with MAbs specific to each receptor. Thus, the process of differentiation from logarithmic-phase to metacyclic promastigotes does not result in a preferential adhesion of the parasites to either one of these complement receptors.

Our studies on both macrophages and cotransfected CHO cells indicate that Mac-1 and CR1 cooperate in the binding of complement-opsonized *L. major* promastigotes. This cooperativity was demonstrated by the requirement for the simultaneous presence of MAbs to both Mac-1 and CR1 to efficiently inhibit serum-dependent adhesion of metacyclic promastigotes to cells expressing both these receptors. The addition of Mac-1 to monocyte-derived macrophages inhibited the adhesion of metacyclic promastigotes by approximately 50%. The addition of MAb to CR1 had little effect on binding when added alone, but the simultaneous addition of MAbs to both Mac-1 and CR1 inhibited adhesion by approximately 70%. Therefore, while the complement-dependent adhesion of metacyclic promastigotes to monocyte-derived macrophages involves both of these complement receptors, Mac-1 appears to play a quantitatively greater role than CR1.

The majority of these studies were done in the presence of C8-deficient serum, as a source of complement. Serum lacking in C8 was used to prevent complement-mediated lysis, thereby preventing potential problems associated with the increased susceptibility of logarithmic-phase organisms to complement-mediated lysis (5, 37). In the present studies, logarithmic-phase organisms consistently bound to complement receptors better than did the metacyclic organisms. The reasons for the increased adhesion of logarithmic-phase promastigotes to both Mac-1 and CR1 are not clear, but one potential explanation is that the lower motility level of the logarithmic-phase organisms allows them to be more easily captured by the purified or recombinant complement receptors used in these studies. Consistent with our previous findings (5, 31), we did not observe any significant direct serum-independent binding of either metacyclic or logarithmic-phase promastigotes to purified complement receptors or to complement receptors expressed on transfected CHO cells.

In the model system with cells transfected with recombinant complement receptors, the absolute numbers of promastigotes adhered to each receptor may not be biologically relevant. For example, complement-opsonized promastigotes bound somewhat better to CHO cells expressing CR1 than they did to cells expressing Mac-1. These observed differences, however, are probably not meaningful. The multiple ligand-binding sites for C3b and C4b on CR1 (20) and the potential heterogeneity of the activation state of the recombinant Mac-1 used in these studies (9, 31) make quantitative binding comparisons between these receptors difficult. Thus, the relative importance of each of these receptors for promastigote adhesion to macrophages cannot be deduced from the extent to which promastigotes bound to the recombinant or purified receptors used in these studies.

On macrophages, the relative contribution of these two receptors could be determined by using blocking MAbs to each receptor. In the presence of serum, promastigote adhesion to

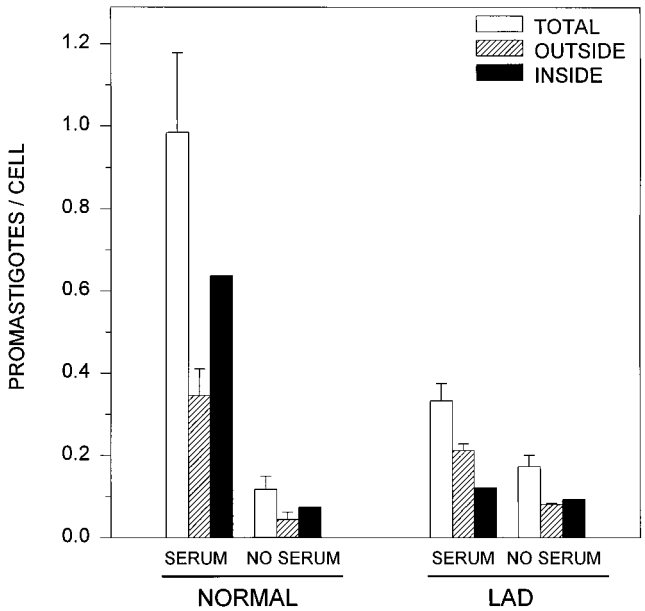

FIG. 9. The phagocytosis of metacyclic *L. major* promastigotes by normal bovine and BLAD monocytes. Metacyclic promastigotes (2.5×10^6 or 3.75×10^6) were added to monolayers of normal or BLAD monocytes, respectively, in either the presence or absence of 4% fresh normal bovine serum as a source of complement. After a 1-h incubation at 35°C, unbound promastigotes were removed by washing, and parallel monolayers were fixed in either methanol or paraformaldehyde. Monocyte-associated *L. major* was stained by indirect immunofluorescence and visualized by fluorescence microscopy. The number of organisms (± SD) visualized in paraformaldehyde-fixed monolayers (outside) was subtracted from the number of organisms in methanol-fixed monolayers (total) to obtain the number of organisms inside. This assay was done in triplicate and is representative of two experiments.

macrophages was markedly inhibited by MAb to Mac-1 but not by MAb to CR1. Serum contains factor I which may skew the adhesion toward Mac-1 by cleaving promastigote-bound C3b to iC3b. To examine this possibility, promastigotes were preopsonized with complement, washed free of serum, and then added to transfected cells or to purified complement receptors in the presence or absence of factor I. This preopsonization system had the added advantage that adhesion could be examined under conditions that did not permit the continuous deposition of new C3b from serum onto the surface of promastigotes. In these assays, the adhesion of serum-opsonized metacyclic *L. major* promastigotes to CR1 was dramatically inhibited by the addition of exogenous factor I. The most likely interpretation of these results is that factor I utilizes the cofactor activity of CR1 to cleave C3b on the promastigotes' surface to an inactive form of the molecule which exhibits decreased affinity for CR1. This decreased affinity allows the promastigotes to be released from CR1 by gentle washing. Thus, by virtue of its factor I cofactor activity, CR1 may actually facilitate the generation of ligands for Mac-1. The transient adhesion of promastigote-associated C3b to CR1 that we observed led us to hypothesize that Mac-1 was the predominant receptor involved in the stable adhesion of complement-opsonized promastigotes to macrophages during receptor-mediated phagocytosis.

To test this hypothesis, we examined the role of these two complement receptors in mediating the complement-dependent phagocytosis of metacyclic promastigotes. In contrast to promastigote adhesion, which utilizes both Mac-1 and CR1, the internalization of bound organisms appears to be a process that is mediated predominantly by Mac-1. Blocking CR1 did not alter the phagocytosis of complement-opsonized promastigotes, whereas blocking Mac-1 significantly diminished phagocytosis. The simultaneous addition of MAbs to both Mac-1 and CR1 resulted in no further inhibition of phagocytosis, relative to that observed with MAb to Mac-1 alone. These results indicate that Mac-1 is sufficient to mediate the uptake of complement-opsonized promastigotes and that CR1 contributes only slightly, if at all, to this process.

To further examine the role of these two receptors during complement-dependent phagocytosis, bovine monocytes from calves with BLAD were studied and compared with normal bovine monocytes. The addition of serum to wells containing normal bovine monocytes resulted in a dramatic increase in the total number of organisms associated with the monocytes and in the number of organisms internalized by the monocytes. In contrast, the addition of serum only marginally increased the total number of promastigotes associated with monolayers of BLAD monocytes and produced no increase in the number of parasites found within BLAD monocytes. Thus the increased phagocytosis of complement-opsonized promastigotes by normal bovine monocytes is a Mac-1-dependent process. Therefore, the complement-dependent phagocytosis of metacyclic promastigotes by both human monocyte-derived macrophages and bovine monocytes appears to be mediated predominantly by Mac-1.

These studies should help to resolve apparent discrepancies regarding the relative importance of Mac-1 and CR1 to promastigote adhesion and subsequent phagocytosis by macrophages. We demonstrate that both Mac-1 and CR1 can participate in the complement-dependent adhesion of metacyclic promastigotes to human monocyte-derived macrophages, and that Mac-1 appears to play a quantitatively greater role than CR1 in this process. Furthermore, Mac-1 appears to be the predominant complement receptor involved in the complement-dependent internalization of metacyclic promastigotes by macrophages.

ACKNOWLEDGMENTS

We thank John Gibas for the flow cytometry analysis.

This work was supported by Public Health Service grant AI24313 from the National Institutes of Health. F.S.S. was supported by the Temple University Medical School M.D./Ph.D. Program.

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Editor: J. M. Mansfield