Leishmania Promastigotes Require Opsonic Complement to Bind to the Human Leukocyte Integrin Mac-1 (CD11b/CD18)

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Abstract. Previous reports have suggested that *Leishmania spp.* interact with macrophages by binding to Mac-1 (CD11b/CD18), a member of the leukocyte integrin family. To better define this interaction, we tested the ability of leishmania promastigotes to bind to purified leukocyte integrins and to cloned integrins expressed in COS cells. We show that leishmania promastigotes bind to cellular or purified Mac-1 but not lymphocyte function-associated antigen-1 in a specific, dose-dependent manner that requires the presence of serum. Binding is inhibited with specific monoclonal antibodies to Mac-1. In the absence of complement opsonization, three different species of leishmania tested fail to bind directly to any of the three leukocyte integrins. We show that binding to Mac-1 requires the third component of complement (C3). Organisms incubated in heat-inactivated serum or serum that has been immunologically depleted of C3 fail to bind to Mac-1. Because the addition of purified C3 to C3-depleted serum restores leishmania binding to Mac-1, we suggest that parasites gain entry into macrophages by fixing complement and subverting a well-characterized adhesive interaction in the immune system between Mac-1 and iC3b.

THE integrins comprise a family of structurally related cell surface receptors that coordinate a network of cell-cell and cell-extracellular matrix interactions (Springer, 1990; Hemler, 1990). Lymphocyte function-associated antigen-1 (LFA-1;[1] CD11a/CD18), Mac-1 (complement receptor type 3 [CR3]; CD11b/CD18), and p150,95 (CD11c/CD18) constitute a subfamily of integrins known as the leukocyte integrins (Kishimoto et al., 1989). These related glycoproteins, which are critical for the adhesive events in the immune system, structurally share a common β subunit that is noncovalently associated with unique α chains.

LFA-1, which is expressed on all leukocytes, mediates lymphocyte adhesion to endothelial cells (Dustin and Springer, 1989), facilitates the T cell conjugation that is required for antigen-specific killer and helper functions (Davignon et al., 1981), and promotes the adhesion required for natural killing (Krensky et al., 1983). These adhesive interactions occur via at least two characterized counter receptors, ICAM-1 (Rothlein et al., 1986; Marlin and Springer, 1987) and ICAM-2 (Staunton et al., 1989; de Fougerolles et al., 1991). p150,95 is restricted in distribution to myeloid cells and a small population of T and B lymphocytes. Its function is not clear, but previous studies have suggested its involvement in the binding of iC3b-opsonized particles (Myones et al., 1988), in the adhesion of cells to endothelium (Keizer et al., 1987*a*,*b*; te Velde et al., 1987), and in cellular adhesion to substrates coated with serum proteins (Anderson et al., 1986; Loike et al., 1991). Mac-1 is primarily expressed on myeloid and natural killer cells, and is responsible for myeloid cell adhesion to endothelial cells, neutrophil homotypic aggregation, and macrophage phagocytosis of foreign particles that are opsonized by complement (Kishimoto et al., 1989). Mac-1 achieves these functions by binding to a multiplicity of cellular and soluble ligands, including ICAM-1 (Smith et al., 1989; Diamond et al., 1990), iC3b (Beller et al., 1982; Wright et al., 1983), fibrinogen (Altieri et al., 1988, Wright et al., 1988), and factor X (Altieri and Edgington, 1988).

Recently, several groups have reported that leukocyte adhesion receptors on macrophages can mediate the direct recognition of microbes in the absence of exogenous complement. Monoclonal antibodies to one or a combination of the leukocyte integrins have been shown to inhibit the phagocytosis of a diverse array of unopsonized microbial pathogens. These include *Leishmania major* (Mosser and Edelson, 1985; DaSilva et al., 1989; Cooper et al., 1988), *Leishmania donovani* (Blackwell et al., 1985), *Leishmania mexicana* (Talamus-Rohana et al., 1990), *Bordatella pertussis* (Relman et al., 1990), *Histoplasma capsulatum* (Bullock and Wright, 1987), *Saccharomyces cerevisia* (Ross et al., 1985; Tamato et al., 1989), *Escherichia coli* (Wright and

1. *Abbreviations used in this paper*: C3, third component of complement; C8D, serum from an individual genetically deficient in the eighth component of complement; CR3, complement receptor type 3; LFA-1, lymphocyte function-associated antigen-1; LPG, lipophosphoglycan.

Jong, 1986), and *Mycobacteria leprae* (Schlesinger and Horwitz, 1990). In some of these studies, macrophages bound directly to these organisms in vitro in the absence of exogenous complement, and this binding was inhibited with antibodies to the CD18 family of adhesion receptors. The mechanism(s) for this direct recognition remains unclear. Mac-1 has been implicated in the binding of saccharides (Ross et al., 1985) and polysaccharides (Wright and Jong, 1986) which may be present on the surface of various microbes. Mac-1 is also reported to bind directly to two leishmania surface structures, the lipophosphoglycans (Talamus-Rohana et al., 1990) and the major surface protease gp63 (Russell and Wright, 1988). In contrast, others (Ezekowitz et al., 1985; Blackwell et al., 1985) have argued that the recognition of microbes by Mac-1 depends on C3, which may be derived from the macrophage itself. To address whether the leukocyte integrins bind directly to endogenous parasite surface structures, or indirectly via complement components, we have investigated the binding of leishmania to transfected fibroblasts expressing leukocyte integrins and to purified leukocyte integrin substrates. In this work we show that the human leukocyte integrins do not support leishmania binding directly, but rather require opsonic complement. Furthermore, we show that when complement is present on *L. major*, the binding of these organisms is mediated almost exclusively by the Mac-1 integrin.

Materials and Methods

Leishmania

The U.S. National Institutes of Health S strain of *L. major* (Bjorvatn and Neva, 1979) was isolated from a West African patient with cutaneous leishmaniasis and was originally provided by Dr. D. Wyler, Tufts University Medical Center (Boston, MA). The Josefa strain of *L. mexicana amazonensis* (Mosser et al., 1986) was isolated from a Brazilian patient with persistent cutaneous leishmaniasis and was provided by Dr. Janet Keithly, New York State Public Health Center (Albany, NY). The 1S strain of *L. donovani* (Murray, 1981) was isolated from a Sudanese patient with kala azar and was provided by Dr. Henry Murray, Cornell Medical School, New York. Promastigotes were cultured in Schneider's complete drosophila medium to stationary phase as described (Mosser et al., 1986) and radiolabeled with tritiated uracil overnight as described (Mosser and Edelson, 1985).

Monoclonal Antibodies

The following murine monoclonal antibodies (mAbs) were used diluted from ascites: LPM19c (IgG2a, anti-CD11b, a gift of Dr. K. Pohlford, Oxford, England) (Uciechowski and Schmidt, 1989), 14B6E.2 (IgG1, anti-CD11b, a gift of Dr. L. K. Ashman, Adelaide, Australia) (Uciechowski and Schmidt, 1989), 904 (IgG1, anti-CD11b, a gift of Dr. J. Griffin, Boston, MA) (Letvin et al., 1983), and TS1/22 (IgG1, anti-CD11a) (Sanchez-Madrid et al., 1982). The following mAbs were used as purified IgG: R6.5 (IgG2a, anti-CD54, a gift of Dr. R. Rothlein, Boehringer-Ingelheim, Ridgefield, CT), YZ.1 (IgG1, anti-CR1, a gift of Dr. R. Jack, Boston, MA) (Changelian et al., 1985), LM2/1 (IgG1, anti-CD11b) (Miller et al., 1986), 60.3 (IgG2a, anti-CD18) (Beatty et al., 1983), OKM1 (IgG2a, anti-CD11b) (Wright et al., 1983), IB4 (IgG2a, anti-CD18) (Wright et al., 1983), and SHCL3 (IgG2b, anti-CD11c) (Schwarting et al., 1985).

Monocyte-derived Human Macrophages

Monocytes were isolated from peripheral blood using "Lymphoprep" (Nycomed Pharma, Oslo, Norway) according to the manufacturer's instructions. They were incubated in RPMI 1640 supplemented with penicillin G/streptomycin (100 U/ml and 100 μg/ml, respectively), 2 mM glutamine, and 5% autologous human serum, in Teflon beakers for 4 d as described (Speert et al., 1988). Cells were adhered to 13-mm round coverglasses overnight in the presence of 5% autologous serum, washed the following day in serum-free phagocytosis buffer, and used in the leishmania-binding assay.

Integrin-coated Substrates

Human leukocyte integrins were purified as previously described (Diamond et al., 1990; Dustin and Springer, 1989; Stacker and Springer, 1991). Briefly, peripheral blood leukocyte lysates or hairy cell leukemia splenocytes were solubilized in Triton X-100 and the integrins were purified by immunoaffinity chromatography. Peak fractions of immunoaffinity-purified LFA-1, Mac-1, and p150, 95 protein at a starting concentration of 3–5 μg/ml in 1% octyl glucoside were diluted 1:12 in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl, and added to nontissue culture-treated 96-well plates (Flow Laboratories, Inc., McLean, VA) for 90 min at room temperature. Plates were subsequently blocked in PBS, 2 mM $MgCl_2$, 1% BSA for 1 h at 37°C and stored at 4°C until further use in the presence of 0.025% NaN_3 and 50 μg/ml gentamicin.

Leishmania-binding Assay

Radiolabeled leishmania promastigotes were resuspended in phagocytosis buffer which consists of equal parts of DME and TC199 buffered with 25 mM Hepes supplemented with 1% BSA. Parasites were added to either purified integrins on 96-well plates or to macrophages or transfected COS cells on 24-well plates for 60 min at 37°C. For assays performed in the presence of serum, a final concentration of 5% serum from a patient with a deficiency in the eighth component of complement, C8D (Mosser and Edelson, 1987), was added to 5×10^7 parasites for 15 min before their addition to cells or to Mac-1–coated substrates. C8D was used to avoid experimental artifacts due to complement-mediated lysis, which causes the release of free uracil from radiolabeled parasites (Mosser and Edelson, 1984*b*). After a 1-h incubation with radiolabeled parasites, plates were washed extensively using a 25-gauge needle aspiration apparatus with warm (35°C) phagocytosis buffer. Bound parasites were solubilized in 0.5% Triton X-100 and 0.1 M NaOH. After neutralization with 0.1 M HCl, lysates were analyzed in a tri-carb scintillation counter (Packard Instrument Co. Inc., Downers Grove, IL).

To inhibit parasite binding to Mac-1 plates, purified lipophosphoglycan (LPG) from *L. major* was added to Mac-1 plates at a final concentration of 100 μg/ml for 15 min before and during the addition of leishmania. To inhibit leishmania C3 opsonization, increasing concentrations of LPG were added to a total of 1×10^7 promastigotes in 5% C8D for 15 min. LPG (McConville et al., 1987) was a gift of Emanuela Handman (Melbourne, Australia).

COS Cell Transfections

COS cells were transfected in 10-cm tissue culture–treated plates with CD11 and CD18 cDNA as previously described (Diamond et al., 1990). Briefly, cells at ~50–60% confluency were transfected with 6 μg each of CD11 and CD18 cDNA by the DEAE-Dextran method for 4 h at 37°C (Kingston, 1987). 72 h after transfection, cells were removed from tissue culture plates with PBS containing 5 mM EDTA and plated onto 13-mm round coverslips in 24-well plates overnight. Cells were assayed the following day, 4 d after transfection. Flow cytometry showed that ~40–60% of LFA-1–transfected cells were positive for LFA-1 expression, and that 30–40% of Mac-1–transfected cells were positive for the expression of Mac-1.

Flow Cytometry

Indirect immunofluorescence and flow cytometry were performed as previously described (Diamond et al., 1990).

SDS-PAGE

Protein samples were analyzed on reducing (5% β-mercaptoethanol) SDS–5% polyacrylamide gels (Laemmli, 1970) and silver stained as described (Diamond et al., 1990).

Results

Leishmania Promastigotes Bind to Human Monocyte-derived Macrophages

Increasing concentrations of radiolabeled *L. major* promastigotes were added to human monocyte-derived macrophages taken from three donors in either the presence or ab-

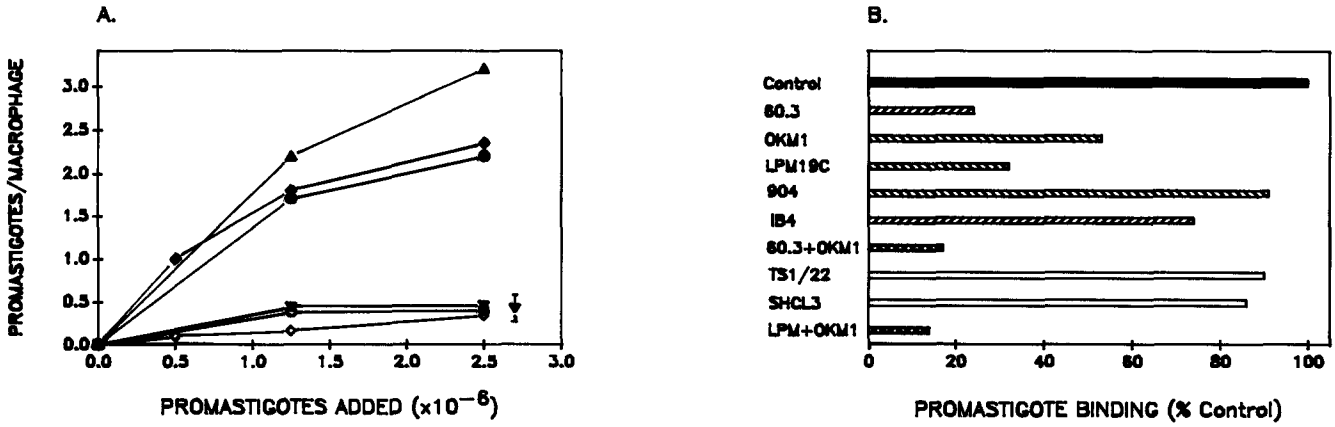

Figure 1. The binding of radiolabeled *L. major* promastigotes to human monocyte-derived macrophages. (*A*) Dose–response curve of *L. major* binding to human macrophages. Increasing concentrations of leishmania promastigotes were added to 24-well plates containing ~5 × 10^4 macrophages per well. In this representative experiment (of six donors, each analyzed twice), cells from three different donors (○, ◇, and △) were incubated with promastigotes in either the presence (*closed symbols*) or absence (*open symbols*) of 5% fresh autologous serum. Standard deviations are shown if they are larger than symbols. Macrophages from one individual (▼) were pretreated for 15 min with 10 μM ATP before the addition of 2.5 × 10^6 promastigotes under serum-free conditions. (*B*) The inhibition of promastigote binding to human macrophages by mAbs to Mac-1. Monocyte-derived macrophages were preincubated with either a 1:100 dilution of ascites (LPM19C, 904, TS1/22) or 10 μg/ml purified antibody (60.3, SHCL3, OKM1, IB4) for 20 min at 35°C. A total of 2 × 10^6 radiolabeled promastigotes were then added for 60 min at 35°C. Counts per minute were determined in triplicate and compared to simultaneous control counts per minute (no antibody). Results reported here are the mean percent of at least two experiments done in triplicate.

sence of 5% fresh human C8-deficient serum (C8D). A parasite dose–response curve (Fig. 1 *A*) shows that promastigotes bind to these cells more efficiently in the presence of serum than in its absence. At the highest parasite input concentration (2.5 × 10^6), which in this experiment corresponds to a parasite/macrophage ratio of 50:1, macrophages in the presence of serum bound an average of between two and three parasites per cell. In the absence of serum at this dose, these macrophages bound <0.5 parasites per cell. The three donors depicted in Fig. 1 were representative of a total of six donors examined. Five of the six donors showed a comparable lack of binding in the absence of serum at all doses tested, and at least a fivefold increase in binding in the presence of serum. A single donor showed greater (approximately twofold) serum-independent binding relative to the other donors tested in parallel. The low binding of *L. major* to human macrophages in the absence of exogenous complement was not improved by pretreating the monolayer with agents that activate Mac-1, including either 0.2 μg/ml PMA (data not shown) or 10 μM ATP (Altieri and Edgington, 1988) (Fig. 1 *A*). The inefficient binding in the absence of serum confirms and extends a previous observation (DaSilva et al., 1989), and establishes that the parasites used in this study were in the infective stage.

Most of the serum-dependent binding of promastigotes to human macrophages was inhibited by mAbs to Mac-1, but not by antibodies to either of the other two leukocyte integrins (Fig. 1 *B*). OKM1, a Mac-1 α chain–specific mAb, inhibited binding by ~50%. The β chain–specific antibody 60.3 inhibited binding by 75%, and a combination of either two Mac-1 α chain antibodies (LPM19C and OKM1) or an α and a β (OKM1 and 60.3) inhibited binding by >80%. Antibodies to either LFA-1 (TS1/22) or p150,95 (SHCL3) inhibited binding by <15%. An antibody to CR1, YZ.1, also had little inhibitory effect in this system (data not shown).

Leishmania Promastigotes Adhere Only to Immobilized Mac-1

Purified leukocyte integrins were isolated by immunoaffinity chromatography and immobilized on plastic. The three integrins studied contain a common M_r 95,000 β chain which noncovalently associates with three α subunits of 177,000, 165,000, and 150,000 M_r (CD11a–c) (Fig. 2). Slight differ-

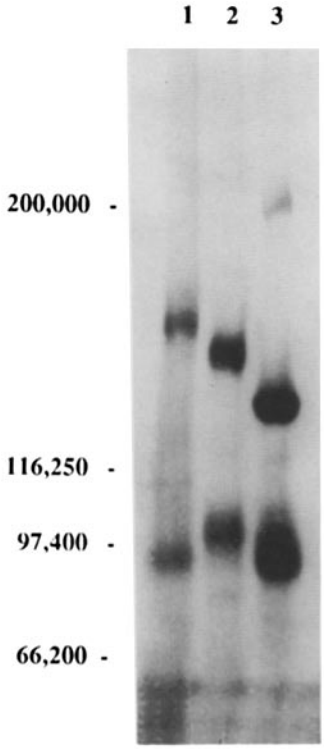

Figure 2. Silver-stained SDS-PAGE of affinity-purified leukocyte integrins. Purified LFA-1 (15 μl) from a TS2/4 mAb affinity column, purified Mac-1 (15 μl) from an LM2/1 mAb affinity column, and purified p150,95 (10 μl) from a CBR-p150/4G1 mAb affinity column (lanes *1*, *2*, and *3*, respectively), were subjected to reduction, SDS–5% PAGE and silver staining. Molecular weight standards are marked to left.

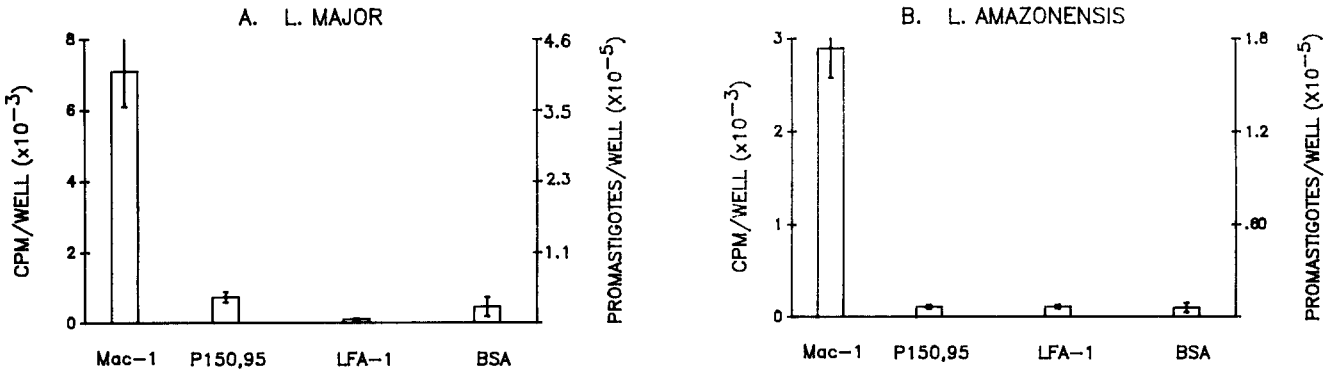

Figure 3. The binding of leishmania promastigotes in 5% serum to plates coated with purified human leukocyte integrins. A total of 2 × 10^6 radiolabeled (*A*) *L. major* or (*B*) *L. amazonensis* promastigotes were added to 96-well plates coated with purified Mac-1, p150,95 or LFA-1 as described in Materials and Methods. After washing the average cpm/well ± SD of triplicate determinations (left axis) and the deduced average number of organisms/well (right axis) were determined. The data shown here for *L. major* are representative of three separate experiments, and that for *L. amazonensis* are representative of two experiments.

ences in the relative migration of the β chains shown in this silver-stained SDS-polyacrylamide gel occur because differential subunit association affects the site-specific glycosylation of the common β chain (Dahms and Hart, 1986). These isolated receptors retain their immunologic epitopes and functional activity. The receptors when adsorbed to plastic retain an active conformation as they bind their respective ligands, including iC3b, ICAM-1, and ICAM-2 (Dustin and Springer, 1989; Diamond et al., 1989; de Fougerolles et al., 1991), without any addition of exogenous stimuli. They also bind mAbs against activation-specific neoepitopes (Diamond, M. S., and T. A. Springer, unpublished observations).

Binding of promastigotes in 5% serum occurred to a significant level with Mac-1–coated substrates (Figs. 3 and 4). The parasites did not bind efficiently to either p150,95 or LFA-1, even though integrin site densities used were able to sustain stimulated endothelial cell adhesion when tested in parallel (data not shown). The binding of parasites to purified Mac-1 was strong, as 20% of the input of *L. major* adhered to Mac-1, whereas 2–5% bound to p150,95 and 1% attached to LFA-1 or the control BSA. At the maximum parasite input (2 × 10^6 parasites/well) the total number of organisms bound to Mac-1 plates in four independent assays was 4.6 × 10^5 ± 3.5 × 10^4 organisms per well, forming a near-confluent lawn of parasites which adhere to the Mac-1 substrate despite the high shear forces created by the washing procedure (Fig. 4). This pattern of Mac-1–dependent binding was reproduced in a second leishmania species, *L. amazonensis* (Fig. 3 *B*).

In the presence of serum, leishmania bind to plates coated with Mac-1 in a parasite dose-dependent manner (Fig. 5 *A*). In this experiment between 20 and 25% of the total organisms added to the plates at each dose remained adherent to the wells after washing. Under serum-dependent conditions in four independent assays, the percentage of the parasite input which adhered to Mac-1 plates ranged from 12 to 26%, with a mean of 20%. In the absence of serum, the number of parasites bound to the Mac-1–coated substrate was similar to that bound to BSA-coated plates.

Parasite binding to Mac-1 plates was also dependent on the

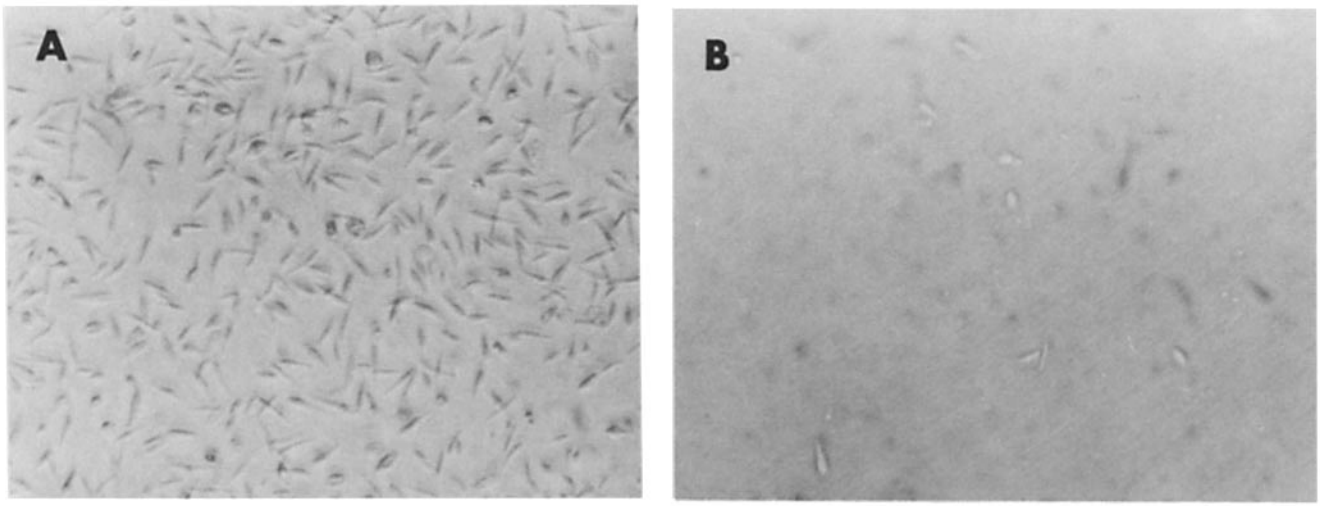

Figure 4. Photomicrograph of *L. major* promastigotes bound to Mac-1–coated plates. A total of 2.5 × 10^6 radiolabeled leishmania were added to purified Mac-1– or LFA-1–coated plates in the presence of 5% serum for 60 min at 35°C. This inverted micrograph shows viable promastigotes adhered tightly to a Mac-1 plate (*A*) but not to LFA-1 (*B*) after extensive washing.

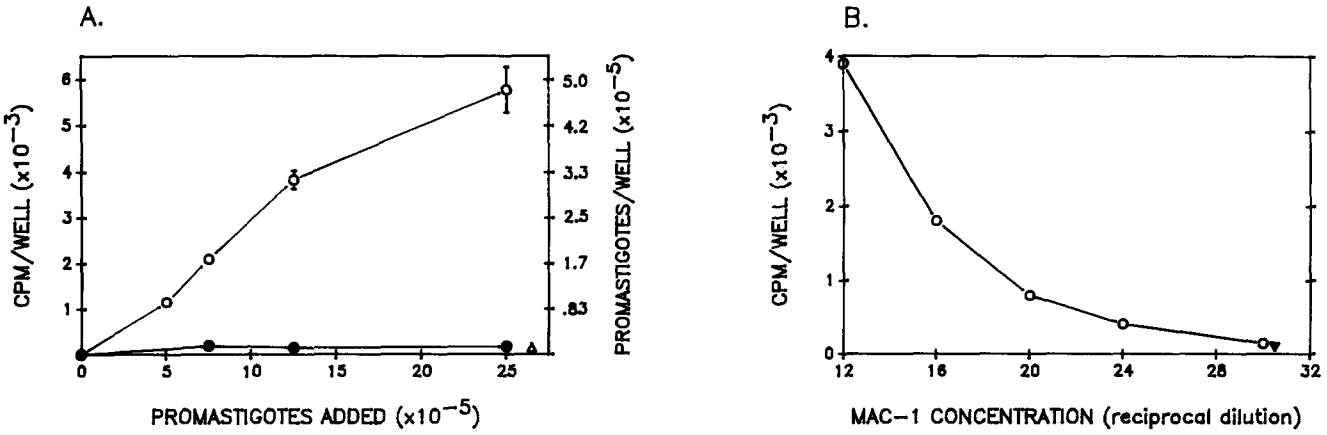

Figure 5. The binding of *L. major* promastigotes to purified Mac-1-coated plates. (*A*) Increasing concentrations of radiolabeled leishmania in the presence (○) or absence (●) of 5% C8D were added for 1 h at 35°C to 96-well plates coated with Mac-1. These data are representative of two experiments. Data points represent the average of triplicate determinations ± SD which are shown only when larger than the symbols. In this experiment leishmania incorporated 0.012 cpm/organism, thus the maximal binding of 5,771 cpm equals a total of 4.8 × 10^5 leishmania bound per well. Background binding of leishmania to parallel BSA-coated wells, which was 91 cpm at the highest parasite input dose (△), was not subtracted from any of the values. (*B*) The binding of leishmania promastigotes to plates coated with decreasing amounts of human Mac-1. A constant amount of radiolabeled *L. major* promastigotes (2 × 10^6) was added to plates coated with increasing dilutions of purified Mac-1 antigen. Mac-1 was diluted from 1:12 to 1:30 in 50 mM Tris, 150 mM NaCl, 2 mM MgCl, which corresponds to a ten-fold range of Mac-1 site densities. A 1:12 dilution of Mac-1 was previously determined to give maximal parasite binding. The binding of leishmania to control wells coated with BSA is included (▼).

quantity of the Mac-1 substrate. A constant amount (2 × 10^6) of leishmania were added to plates, with increasing dilutions of Mac-1 immobilized to plastic wells (Fig. 5 *B*). Over a range of Mac-1 dilutions, parasite binding decreased from a maximum of 2.3 × 10^5 parasites/well to levels indistinguishable from background (Fig. 5 *B*).

Adhesion of Leishmania to Mac-1 Plates Requires Opsonic Complement

The binding of promastigotes to Mac-1 plates is dependent on serum complement. Three different species of leishmania—*L. major*, *L. amazonensis*, and *L. donovani*—were added to Mac-1 plates either in the presence of C8D, in the presence of heat-inactivated C8D (56°C, 60 min), or in the absence of serum. All the species tested bound to Mac-1 plates only when fresh serum containing opsonic complement was present (Fig. 6, *A–C*). In the case of *L. major*, either heat inactivation of serum or immunologic depletion of C3 from serum decreased binding from peak levels (10,259 cpm) to background (289 and 364 cpm, respectively). Furthermore, pretreatment of serum with agents that are known to biochemically inactivate C3 or prevent C3 deposition inhibited parasite binding to Mac-1 by >80% (Table I). The same trend was observed with the other two species tested as well. The binding of *L. amazonensis* to Mac-1 in the presence of 5% C3-depleted serum (C3D) was 661 ± 206 cpm. The addition of 250 μg/ml of purified C3 to this serum (Quidel, San Diego, CA) partially restored binding to 3,692 ± 1114 cpm (Fig. 6 *B*). In a separate experiment, the addition of purified C3 to C3D partially restored binding of *L. major* from 472 ± 76 cpm to 1,509 ± 346 cpm.

The Binding of Promastigotes to Mac-1 Substrates Is Specific and Inhibited by mAbs

Promastigotes were added to Mac-1 plates which had been preincubated for 20 min with various mAbs (Fig. 7). Two mAbs to Mac-1, LPM19C and 14B6E.2, each inhibited binding by >80%, while a third mAb, LM2/1, inhibited binding by <30%. OKM1 inhibited binding by 53%. A combination of OKM1 and LPM19C inhibited binding to levels indistinguishable from background. An isotype matched antibody R6.5, against a control antigen, ICAM-1, failed to inhibit parasite binding. Interestingly, two mAbs (904 and IB4) which have been reported to inhibit LPG binding to Mac-1 but not affect iC3b binding to Mac-1, had little, if any, effect in our binding assay.

Leishmania LPG Does Not Directly Compete for Mac-1 Binding

Because other groups report a role of leishmania LPG in the binding of leishmania to macrophages (Talamus-Rohana, 1990; Handman and Goding, 1985), we studied its role in our defined system. LPG from *L. major* promastigotes (100 μg/ml) was preincubated with Mac-1-coated plates for 15 min before the addition of increasing concentrations of preopsonized leishmania. LPG did not inhibit parasite binding. Leishmania bound to LPG-treated Mac-1 plates as well as they bound to control plates (Fig. 8 *A*). However, if leishmania were incubated simultaneously with LPG and complement at the time of opsonization, LPG partially inhibited the binding of parasites to Mac-1 in a dose-dependent manner (Fig. 8 *B*). This inhibitory effect was overcome by the addition of excess (20%) serum. In this system, LPG inhibited the binding of leishmania to Mac-1 only when added together with complement, presumably by competing with parasites for complement deposition.

Promastigote Binding to COS Cells Expressing Cloned Leukocyte Integrins

While the adhesion assays of leishmania promastigotes to

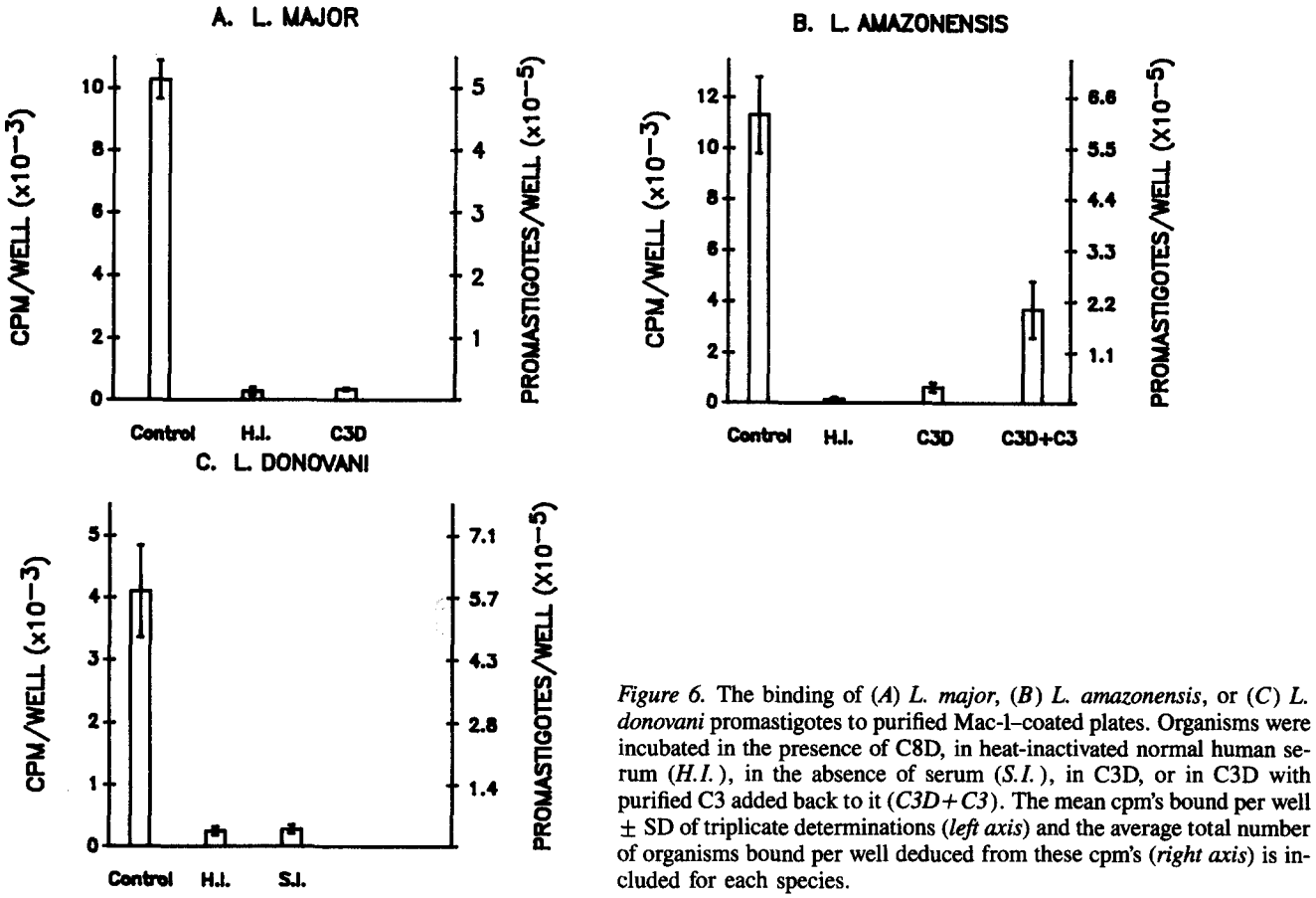

Figure 6. The binding of (*A*) *L. major*, (*B*) *L. amazonensis*, or (*C*) *L. donovani* promastigotes to purified Mac-1–coated plates. Organisms were incubated in the presence of C8D, in heat-inactivated normal human serum (*H.I.*), in the absence of serum (*S.I.*), in C3D, or in C3D with purified C3 added back to it (*C3D+C3*). The mean cpm's bound per well ± SD of triplicate determinations (*left axis*) and the average total number of organisms bound per well deduced from these cpm's (*right axis*) is included for each species.

purified leukocyte integrins strongly suggested an interaction with Mac-1, we wanted to confirm this in a cellular system. To achieve expression of only a single leukocyte integrin, COS cells were cotransfected with cDNAs for the common β subunit and either the Mac-1 or LFA-1 α subunit. These cells were used in a leishmania promastigote–binding assay 4 d after transfection. At this time surface expression of LFA-1 ranged from 40 to 60%, while the expression of Mac-1 ranged from 30 to 40%, as determined by flow cytometry (data not shown; Diamond et al., 1990). A range of leishmania doses was added to these cells in either the presence or absence of exogenous complement.

COS cells transfected with Mac-1 showed greater binding of parasites than did mock-transfected cells (Fig. 9). In contrast, the binding of *L. major* to LFA-1–transfected COS cells was similar to that of *L. major* to mock-transfected cells. The increased binding of leishmania to Mac-1–expressing COS cells, as in the purified protein experiments, required the presence of serum. This increase in parasite binding was specific to Mac-1, as an antibody to Mac-1 but not to LFA-1 inhibited binding to levels obtained without serum. Microscopic observation (Fig. 10) reveals that between 30 and 40% of the cells bound larger numbers of parasites (>10), whereas the remaining cells bound only 1–2 organisms. This fraction of the cells which sustain strong parasite binding correlates well with the profile of Mac-1 expression seen by flow cytometry and suggests that cells expressing high amounts of Mac-1 bind significantly greater numbers of parasites than do transfected cells which do not express significant levels of Mac-1.

Discussion

Leishmania are obligate intracellular parasites that reside almost exclusively within mononuclear phagocytes (Chang, 1983). Several groups have examined the molecular basis for the binding and internalization of promastigotes by macrophages in vitro and have demonstrated that specific macrophage phagocytic receptors mediate this interaction (Mosser and Edelson, 1984*b*; DaSilva et al., 1989; Blackwell et al., 1985; Wilson and Pearson, 1988; Chang, 1983; Talamus-Rohana et al., 1990). Previous studies have shown that leishmania are able to fix complement directly from nonimmune

Table I. The Binding of Radiolabeled Leishmania to Mac-1 Plates after Incubations in Serum Depleted of C3

Treatment*	cpm ± SD‡	Inhibition
		%
Untreated	2,402 ± 268	–
CoVF	471 ± 8	80
KSCN	236 ± 106	90
Hydroxylamine	491 ± 289	80
EDTA	216 ± 13	91
C3D	207 ± 5	91
No serum	219 ± 18	91

* C8D was pretreated for 30 min at 35°C with 5 U/ml cobra venom factor, 0.2 M potassium thiocyanate, 0.2 M hydroxylamine, or 15 mM EDTA.
‡ Counts per minute of radiolabeled leishmania bound to Mac-1 plates after washing ± standard deviation of triplicate samples.

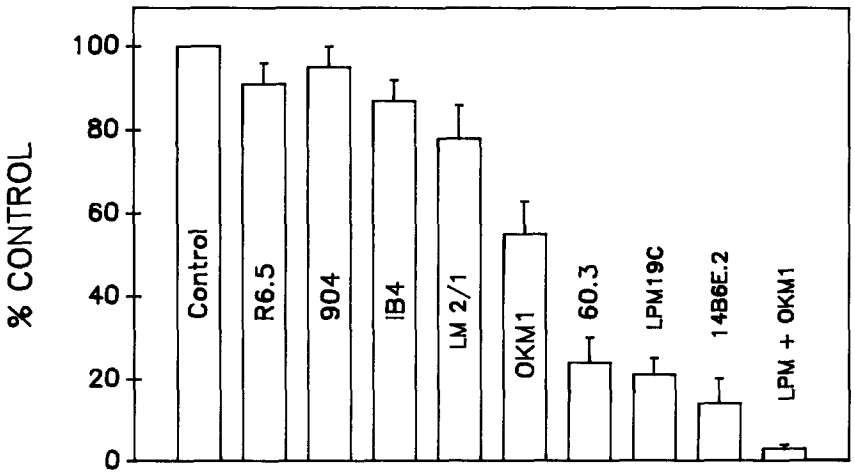

Figure 7. The inhibition of *L. major* promastigote binding to Mac-1 substrates. Mac-1 substrates were incubated with mAbs for 20 min before and during the addition of 2×10^6 leishmania in 5% C8D for 60 min at 35°C. Antibodies LPM19C, 904, and 14B6E.2 were used as ascites diluted 1:100. Antibodies R6.5, IB4, LM2/1, and OKM1 were used as purified IgG at a final concentration of 10 μg/ml. R6.5 mAb to ICAM-1 was used as a nonbinding control antibody. Counts per minute were determined in triplicate and compared to simultaneous control counts per minute (no antibody). Results reported here are the mean percent control of at least two experiments done in triplicate.

serum (Mosser and Edelson, 1984*b*; Puentes et al., 1988), and that this enhances entry into (Mosser and Edelson, 1985) and survival within macrophages (Mosser and Edelson, 1987). This increased entry requires complement and is greatly reduced by mAbs to CD11b (Blackwell et al., 1985; Mosser and Edelson, 1985). Interestingly, several groups have reported that leishmania bind directly to macrophage receptors in the absence of serum or exogenous complement, and that this interaction is also inhibited by mAbs to Mac-1 (Blackwell et al., 1985; Mosser and Edelson, 1985; Russel and Wright, 1988; Talamus-Rohana et al., 1990; Cooper et al., 1988). Some of these groups (Russell and Wright, 1988; Talamus-Rohana et al., 1990) suggest that leishmania surface molecules may bind directly to Mac-1 and perhaps the other leukocyte integrins. A potential criticism of these experiments is that even in the absence of exogenous serum, macrophages may produce enough endogenous C3 to facilitate a complement-dependent parasite binding (Wozencraft et al., 1986). Thus, it is still difficult to discern whether leishmania binding to macrophages through the leukocyte integrins requires complement.

To address this question, we developed an assay for leishmania binding in which the presence of opsonic complement could be rigorously controlled. Parasites were preincubated in the presence or absence of complement and allowed to adhere to both transfected fibroblasts expressing members of the leukocyte integrin family (Mac-1 and LFA-1) or to immunoaffinity-purified leukocyte integrin substrates (Mac-1, LFA-1, and p150,95). Our results suggest that leishmania promastigotes require opsonic complement to bind to the leukocyte integrins. Furthermore, we find that opsonized leishmania bind predominantly to the Mac-1 integrin. They do not bind appreciably to LFA-1 and bind only weakly to p150,95.

Our evidence for these conclusions is as follows: (*a*) Two species of leishmania (*L. major* and *L. amazonensis*) bound strongly to purified Mac-1 substrates but not to those containing LFA-1 or control proteins. Binding to purified p150,95 was reproducibly weaker, but was partially inhibited by specific mAb (data not shown). All three purified receptors retain their antigenicity and biological function (Dustin and Springer, 1989; Diamond et al., 1990; Stacker and Springer, 1991), and the site densities used in this study were sufficient to sustain endothelial cell binding (data not shown). (*b*) The binding to Mac-1 was dose-dependent both for the concentration of parasites and Mac-1. It was also specific because it was inhibited strongly by some but not all mAbs to Mac-1. (*c*) *L. major* showed an enhanced ability to bind to COS cells expressing Mac-1, but not to LFA-1 or mock-transfected cells. This increase was abolished by mAbs to Mac-1. The low-level background binding of promastigotes to untransfected and LFA-1–transfected COS cells presumably occurred by a serum-independent mechanism which may be mediated via lectin-like receptors on mammalian cells

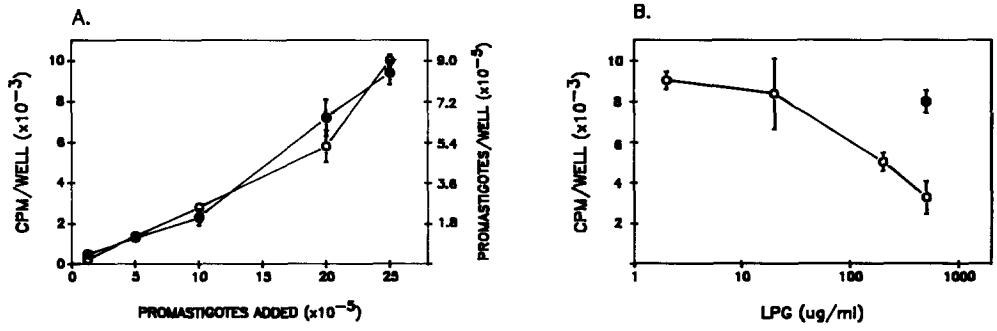

Figure 8. The effect of purified leishmania LPG on the binding of *L. major* promastigotes to Mac-1 plates. (*A*) Increasing concentrations of preopsonized leishmania promastigotes were added to Mac-1 plates which had been pretreated for 15 min with 100 μg/ml purified LPG (•) or saline (○). The LPG was left in the wells during the incubation with leishmania. (*B*) A total of 1×10^7 promastigotes were incubated in 5% C8D serum and increasing concentrations (0–500 μg/ml) of leishmania LPG for 15 min. A constant amount of unwashed promastigotes (2×10^6) was added to untreated Mac-1 plates in triplicate (○). The binding of an equal number of parasites incubated in 20% C8D and 500 μg/ml LPG is included (•).

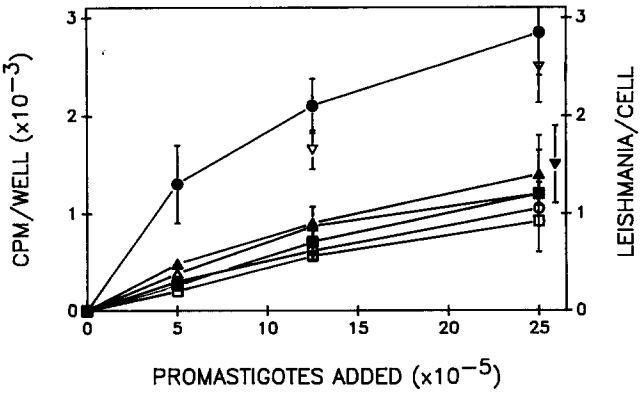

Figure 9. The binding of radiolabeled *L. major* promastigotes to transfected COS cells. Increasing concentrations of promastigotes were added to COS cells transfected with Mac-1 (○,●), with LFA-1 (△,▲), or with plasmid alone (□,■) in the presence (closed symbols) or absence (open symbols) of 5% C8D. Binding of promastigotes in serum to Mac-1 substrates in the presence of antibody to Mac-1 (LPM19C) (▼) or to LFA-1 (TS1/22) (▽) is included. Data points represent the mean (triplicates) cpm/well ± SD (*left axis*) and the deduced number of parasites per cell.

(Blackwell et al., 1985; Wilson and Pearson, 1988) recognizing LPGs on leishmania (Bouvier et al., 1985; Handman and Goding, 1985). (*d*) The binding of leishmania to purified Mac-1 or to COS cells expressing Mac-1 required the presence of complement, since incubation in the absence of serum, in the presence of heat-inactivated serum, or with serum immunologically or biochemically depleted of C3 eliminates binding. The addition of C3 to depleted serum partially restored binding to purified Mac-1. Failure to completely restore binding was due to complement-mediated lysis of promastigotes after C3 addition, as the C3-deficient serum used contains all the terminal components of the complement cascade. Lysed promastigotes release radiolabel (Mosser and Edelson, 1984), which was removed by washing. (*e*) These experiments with transfected and purified Mac-1 agree with data from human macrophages, as the presence of active complement greatly enhances binding of parasites, and this increase was inhibited almost completely by blocking mAbs to Mac-1, and not to LFA-1 or p150,95. Interestingly, in our system, the complement receptor type 1 (CR1) apparently has only a minor role in promastigote binding. Monoclonal antibodies to the CR3 (OKM1 and LMP-19c) block the majority of parasite binding to human macrophages, whereas mAbs to the CR1 block poorly, if at all.

The requirement for opsonic complement for leishmania binding to Mac-1 suggests that the interaction between parasite and macrophage may be mediated by Mac-1 binding to iC3b, a well-characterized adhesive interaction that is critical to immune clearance of foreign particles (Beller et al., 1982; Wright et al., 1983). The mAbs to Mac-1 which inhibit leishmania binding are the same as those which block iC3b binding to cellular or purified Mac-1 (Diamond et al., 1989; Garcia-Aguilar, J., M. S. Diamond, and T. A. Springer, manuscript in preparation): LPM19c and 14B6E.2 block strongly, OKM1 inhibits partially, and LM2/1 blocks only weakly the binding of leishmania to Mac-1. Parasites which bind to intact macrophages without a requirement for exogenous complement may be able to fix the small amount of C3 that is generated endogenously by macrophages. In contrast, others have demonstrated that beads coated with isolated leishmania molecules bound to neutrophils in the absence of complement (Talamus-Rohana et al., 1990). This binding was inhibited by two mAbs (904 and IB4) that react with Mac-1 at sites distinct from the iC3b binding site. We believe that this serum-independent binding represents a minor contribution, because when we use intact viable parasites, these two antibodies have little effect on binding to

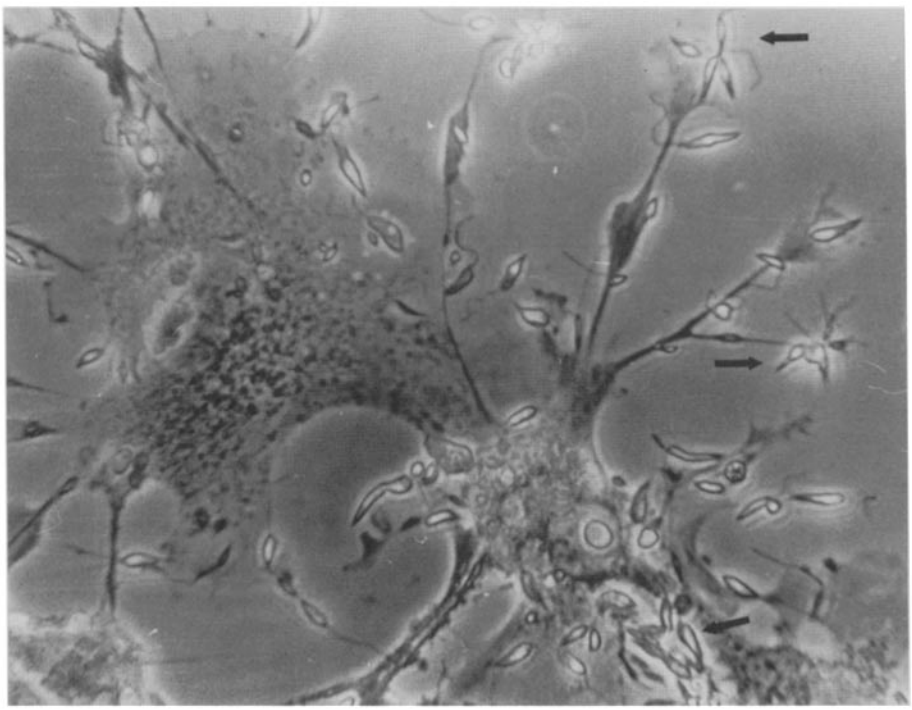

Figure 10. Photomicrograph showing the binding of *L. major* promastigotes to COS cells expressing Mac-1. A total of 2.5 × 10^6 leishmania were added to 24-well plates containing ~5 × 10^4 COS cells in the presence of 5% C8D. Numerous promastigotes bound to the periphery of some cells are indicated by arrows.

Mac-1. The fixation of complement by the two leishmania surface structures gp63 and LPG may explain in part why other groups have observed a role for these structures in the binding of leishmania to macrophages (Talamus-Rohana et al., 1990; Russell and Wright, 1988). In our experiments, the inhibition of leishmania binding to Mac-1 by LPG did not occur unless LPG was coincubated with parasites and complement. This inhibitory effect occurred at relatively high LPG concentrations and was overcome by the addition of excess serum. Thus, we believe that the inhibitory role of soluble LPG in our system is due, at least in part, to its ability to fix and consume complement.

Using purified proteins and transfected cells, we have examined the interaction of leishmania with individual cell surface receptors and conclude that there is a requirement for opsonic complement for parasite interaction with Mac-1. It is interesting that several other microorganisms have been observed to bind to Mac-1 including *E. coli* (Wright and Jong, 1986), *B. pertussis* (Relman et al., 1990), and *H. capsulatum* (Bullock and Wright, 1987). These studies were performed using macrophages as the source of Mac-1. It will be interesting to determine whether these organisms bind directly to Mac-1 or, in a manner similar to leishmania, require opsonic complement to allow adhesion. We hypothesize that many intracellular microbes may subvert the well-characterized Mac-1–iC3b phagocytosis pathway to initiate cellular infections.

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