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Comparison of Receptors Required for Entry of *Leishmania major* Amastigotes into Macrophages

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We investigated the mechanisms of entry of amastigotes of *Leishmania major* from two different sources into macrophages by comparing their use of the Fc receptor (FcR), complement receptor type 3 (CR3), and mannose-fucose receptor (MFR). Amastigotes were obtained from BALB/c mice and SCID mice. FcR involvement was examined by opsonizing *L. major* with parasite-specific immunoglobulin G (IgG). Anti-parasite IgG did not alter the uptake of amastigotes from BALB/c mice since these amastigotes had antibody bound to their surface: IgG1 was the most predominant antibody, followed by IgG2b, IgM, and IgG2a. However, opsonization with antiparasite IgG enhanced the entry of amastigotes that lacked antibody on their surface, namely, amastigotes obtained from SCID mice or from macrophages infected in vitro. These results indicate that the FcR is important for amastigote entry into macrophages. Down-modulation of FcRs onto immune complexes, however, did not reduce the entry of amastigotes containing surface-bound IgG into macrophages. Monoclonal antibodies against the CR3 inhibited the entry of amastigotes from either BALB/c or SCID mice into J774A.1 macrophage-like cells. Simultaneous blocking of FcR and CR3 further increased the inhibition of phagocytosis. Treatment of macrophages with soluble mannan or down-modulating the MFR onto mannan-coated coverslips had no effect on the entry of amastigotes from BALB/c or SCID mice. Thus, the MFR does not appear to be used by amastigotes of *L. major*. We show that ingestion of amastigotes appears to occur primarily through the FcR and CR3; however, additional receptors may also participate in the uptake of amastigotes.

Leishmania spp. are obligate intracellular parasites of macrophages. They have two major developmental forms, namely, the extracellular promastigote that develops in the sandfly and initiates infection in the mammalian host and the obligate intracellular amastigote that multiplies in macrophages. The macrophage is the only cell *Leishmania* spp. inhabit, implying a high degree of specificity for this cell by the parasite. Entry into macrophages involves recognition of specific parasite ligands by receptors on the macrophage surface and eventual internalization of the parasite by the macrophage. Numerous studies have identified macrophage receptors involved in the entry of *Leishmania* promastigotes. These receptors include the mannose-fucose receptor (MFR) (1, 4, 7, 9, 25, 29, 34, 35), the receptor for advanced glycosylation end products (AGE) (21), the fibronectin receptor (27, 37), the Fc receptor (FcR) (8, 28), and the complement receptors CR1 and CR3 (4, 10, 11, 17–20, 30, 33, 35).

Despite the wealth of information about the receptors used for entry of the promastigote stage, very little is known about the mechanisms of entry of amastigotes which are responsible for the disease in mammalian hosts. The aim of the present study was to determine what receptors are used by amastigotes of *Leishmania major* to gain entry into macrophages. Our results suggest both differences and similarities in the macrophage receptors utilized by amastigotes from two sources, BALB/c and SCID mice.

BALB/c mice were purchased from Charles River (St. Constant, Quebec, Canada) and SCID mice (C.B17/lcr-*scid*) were purchased from Taconic Laboratories (Germantown, N.Y.). Six- to 10-week-old SPF-derived male C57BL/6 mice (Taconic Laboratories) were used as a source of bone-marrow-derived macrophages (BMDM). These mice were housed in a P2 barrier facility and routinely tested for bacterial pathogens and subclinical viral infections.

The NIH/173 (WHOM/lr/−/173) strain of *L. major* was used in all studies. Amastigotes were maintained in BALB/c mice by injecting 1×10^6 to 2×10^6 parasites into each hind footpad. The parasites were purified from the footpads as described previously (2). Amastigotes were stained for viability with fluorescein diacetate and ethidium bromide and counted with a hemocytometer (14).

BMDM were obtained by flushing the femurs of mice as previously described (2) and used after 9 to 11 days of culture, when approximately 98% of the cells are macrophages. Resident peritoneal macrophages were obtained by peritoneal lavage with 10 ml of Dulbecco modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, N.Y.) medium containing 10% fetal bovine serum and 50 μg of gentamicin per ml. The J774A.1 macrophage cell line (American Type Culture Collection [ATCC]) was maintained in vitro by serial passage in DMEM containing 10% fetal bovine serum and 50 μg of gentamicin per ml.

The bioassay for the entry of amastigotes into macrophages was performed by using either suspension cultures or macrophages adhered to glass slides. In the suspension assay, macrophages were suspended in DMEM at a concentration of 2.5×10^5 cells per 0.5 ml in polypropylene tubes (Falcon; Becton Dickinson, Lincoln Park, N.J.) and incubated for 2 h at 37°C and 5% CO_2 before use. Test solutions were added (0.1 ml), and the cells were incubated for 30 min at 37°C, after which 5×10^5 amastigotes from BALB/c mice or 2.5×10^6 amastigotes from SCID mice, in 0.1 ml, were added to the macrophages and this mixture was incubated

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for 2 h at 37°C. At these concentrations of amastigotes, we routinely obtained 50% infected macrophages in cultures. After incubation, the cell suspension was centrifuged at 50 × *g* for 7 min, the supernatant was gently removed by aspiration, and the pellet was resuspended in 0.5 ml of DMEM. Cell smears were prepared with a cytocentrifuge (Shandon, Pittsburgh, Pa.) and stained with Wright stain (Leukostat; Fisher Scientific, Orangeburg, N.Y.). The percentage of infected cells and the number of parasites per 200 macrophages were determined by microscopic examination of stained cell smears under oil immersion (2). In the adherent assay, macrophages were added to wells of eight-well Lab-Tek tissue culture chamber slides (Nunc, Naperville, Ill.) that were coated with 0.1 mg of poly-L-lysine (molecular weight, 70,000 to 150,000; Sigma Chemical Co., St. Louis, Mo.) per ml for 1 h at room temperature. Macrophages were allowed to adhere for 1 h, and then the wells were washed twice with DMEM prior to the addition of amastigotes. After a 1-h incubation period, the wells were washed twice with DMEM, and the slides were air dried, fixed, and stained as described above.

The involvement of the FcRs in the entry of *Leishmania* spp. has received little attention. We examined the role of the FcR in the entry of amastigotes of *L. major* by quantifying the entry of parasites opsonized with parasite-specific immunoglobulin G (IgG) into macrophages. Amastigotes were incubated for 30 min in parasite-specific or normal IgG at a concentration of antibody (25 μg/ml) which was found to be nonagglutinating. Excess antibody was removed by centrifugation for 10 min at 250 × *g*. Parasite-specific IgG was prepared from the sera of BALB/c mice obtained 28 to 35 days postinfection with *L. major*. IgG was isolated by using a protein A-agarose column (Sigma) (13). Normal mouse IgG (Sigma) was used as a control.

Chang (8) observed enhanced entry of promastigotes in the presence of immune serum. We confirm his finding that the entry of promastigotes in the presence of parasite-specific IgG was enhanced. The percentage of infected macrophages increased from 34% ± 1% when promastigotes were opsonized with normal mouse IgG to 66% ± 2% when they were opsonized with parasite-specific IgG. However, the entry of amastigotes obtained from BALB/c mice was not enhanced in the presence of parasite-specific IgG, suggesting that the FcR plays no role in amastigote entry. We know, however, that amastigotes obtained from the footpads of BALB/c mice have antibody bound to their surface. The presence of surface-bound IgG was detected by an enzyme-linked immunosorbent assay (ELISA) by using whole parasites as the source of antigen. The parasites were washed three times in sterile phosphate-buffered saline (PBS) and adjusted to 2×10^6/ml, and 100 μl was added to each well of a 96-well plate (Nunc, Kamstrup, Denmark). The plates were kept at 37°C for 3 h to allow the parasite to settle and adsorb to the well. The supernatant was removed, and the plates were air dried at room temperature for 15 to 30 min. Plates were stored at −20°C prior to use in the assays. Isotypes were determined by using the Mouse Typer subisotyping panel (Bio-Rad Laboratories, Richmond, Calif.). The most predominant antibody isotype found on the amastigote surface was IgG1, followed by IgM, IgG2a, and IgG2b in similar proportions (Fig. 1). The antibody pattern on the surface of the amastigote is similar to that observed in the sera of mice infected with *Leishmania tropica* (*L. major*) (24). Thus, the surface of the parasite is saturated with Ig, which may explain why we did not observe enhanced entry

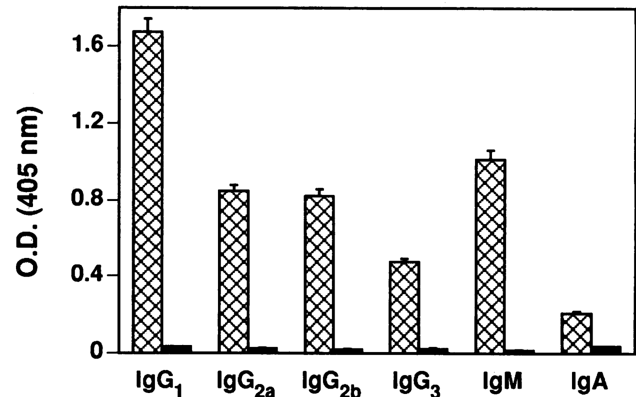

FIG. 1. Isotype distribution of anti-*L. major* antibodies on the surface of amastigotes isolated from BALB/c (▩) and SCID (■) mice. Shown are the mean optical density (O.D.) values ± standard errors from an ELISA. Amastigotes were obtained from five animals per strain of mouse.

of amastigotes after the addition of exogenous parasite-specific IgG to the cultures.

To determine the importance of the FcR in entry, we developed two procedures to obtain amastigotes free of host Ig. Our first source of Ig-free amastigotes was BMDM infected in vitro with promastigotes of *L. major*. Nine- to 12-day-old macrophages were infected and cultured at 37°C for an additional 10 to 12 days to allow the promastigotes to transform into amastigotes and for amastigote replication to occur. The amastigotes were liberated from the infected cells by lysing macrophages with 0.01% sodium dodecyl sulfate and washed prior to use in the assays. Our second source of Ig-free amastigotes was SCID mice infected with *L. major*. SCID mice lack the ability to produce Ig, and the amastigotes from these mice do not have surface-bound Ig as shown by ELISA (Fig. 1). By using these amastigotes, we observed a significant enhancement of parasite entry in the presence of specific IgG (Fig. 2). Opsonization of the amastigotes obtained from in vitro culture and SCID mice

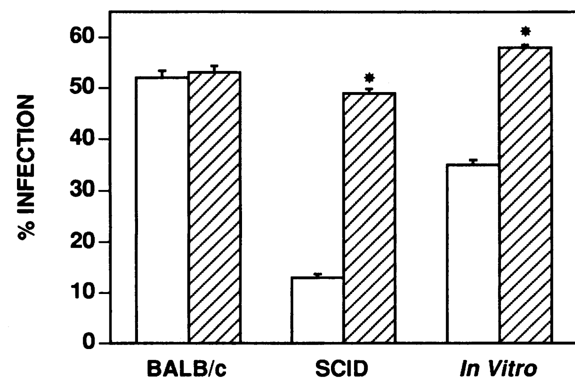

FIG. 2. Entry of amastigotes opsonized with 25 μg of normal (□) or parasite-specific (▨) IgG per ml into BMDM. The ratio of amastigotes to macrophage was 2:1. The sources of amastigotes were the footpads of BALB/c and SCID mice or BMDM cultures in vitro. Results were expressed as the mean percentage of infected macrophages ± standard error of three replicates per group. These data are representative of at least two experiments with similar results. *, significantly different (by analysis of variance; $P < 0.01$).

TABLE 1. Percentage of macrophages adhered to immune complexes infected with *L. major* amastigotes obtained from different sources[a]

Treatment	% Macrophages infected with amastigotes from[b]:	
	BALB/c mice	SCID mice
DNP	74 ± 2	59 ± 3
Anti-DNP	68 ± 4	57 ± 2
Immune complex	67 ± 2	63 ± 1
Trypsinization	62 ± 1	60 ± 3
Immune complex + trypsinization	58 ± 3	64 ± 5

[a] Macrophages were adhered to coated slides for 1 h prior to exposure to amastigotes.

[b] Values are the means ± standard errors of four replicates.

enhanced the uptake of the parasite by 40 and 73%, respectively. These results indicate that the FcR is utilized by amastigotes to enter macrophages.

Binding to the FcR and MFR triggers respiratory-burst activity, whereas binding to the CR1 and CR3 does not (36). It is possible that the FcR would not be an appropriate receptor for amastigotes to use because they would be subjected to the toxic molecules generated by the respiratory-burst activity. In our studies, amastigotes obtained from either BALB/c or SCID mice survived within macrophages in vitro for at least 72 to 96 h. In addition, amastigotes from SCID mice, opsonized with IgG, survived for 72 h within BMDM in vitro. We observed that infection levels in BMDM in vitro, 72 h after exposure to amastigotes from SCID mice, were 70, 80, and 87% for unopsonized amastigotes, IgG-opsonized parasites, and complement-opsonized parasites, respectively. Therefore, amastigotes have mechanisms to evade the toxic molecules generated by the respiratory burst as was reported previously (22).

We then examined the importance of FcRs in the entry of *Leishmania* spp. by down-modulating the receptors onto immune complexes by the procedure of Michl et al. (16). Briefly, poly-L-lysine-coated wells were incubated for 30 min at room temperature with 0.1 ml of a 16.8-mg/ml concentration of human dinitrophenyl (DNP)-albumin (Sigma), washed, and reacted with 0.1 ml of a 200-μg/ml concentration of rabbit anti-DNP (Sigma) for 30 min at room temperature. The macrophages were adhered to the coated slides for 1 h prior to exposure to amastigotes. As expected, we found no change in the percentage of macrophages infected with amastigotes from SCID mice when they were adhered to immune complexes as compared with those adhered to the individual components of the complex, either DNP or anti-DNP (Table 1). Surprisingly, we saw only a slight but not significant decrease in the uptake of amastigotes from BALB/c mice into macrophages adhered to complexes (Table 1). In addition, we did not observe a reduction in the entry of amastigotes from SCID mice that were opsonized with parasite-specific IgG into macrophages adhered to immune complexes, whereas phagocytosis of Ig-opsonized sheep erythrocytes was significantly reduced (not shown). Michl et al. (16) demonstrated that the FcR for IgG2a is not down-modulated onto complexes and that treatment of macrophages with trypsin destroys this receptor. To ensure that amastigotes were not entering by binding the FcγRI, we treated macrophages with trypsin (Table 1). We did not observe a reduction in the uptake of the amastigotes, indicating that the FcγRI for IgG2a is not required for the entry of amastigotes. In addition to using immune complexes to down-modulate Fc receptors, we employed the monoclonal antibody (MAb) 2.4G2, which recognizes the FcγRII. Culturing macrophages onto slides coated with up to 50 μg of MAb 2.4G2 per ml did not alter the uptake of amastigotes obtained from either strain of mouse (data not shown).

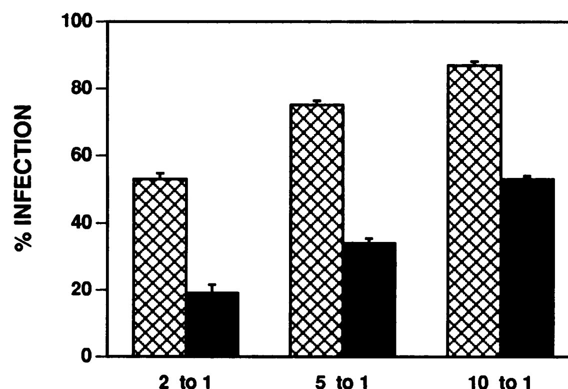

FIG. 3. Entry of amastigotes of *L. major* from the two sources, BALB/c (▩) and SCID (■) mice, into BMDM at different parasite/macrophage ratios. Stained macrophages were examined by microscopy for the percentage of infected cells (200 cells observed per replicate). Results were expressed as the mean ± standard error of three replicates per group.

Although opsonization with IgG, and therefore use of the FcR, enhanced the entry of amastigotes into macrophages, we found that amastigotes also utilize other receptors to enter macrophages. For example, amastigotes from SCID mice, which lack IgG on their surface, are also phagocytosed by macrophages. The uptake of amastigotes from SCID mice, however, is not as efficient when compared with that of amastigotes from BALB/c mice. To obtain an infection rate of 50%, a 2:1 amastigote/macrophage ratio is required when amastigotes from BALB/c mice are used. On the other hand, a 10:1 ratio is necessary to obtain 50% infection when amastigotes from SCID mice are used (Fig. 3). Interestingly, the infectivity of amastigotes obtained from SCID mice resembled more closely that of promastigotes; at a 2:1 ratio, only 10 to 20% of BMDM became infected. Note that the maximum percentage of infected macrophages was approximately 80% when amastigotes were used at a 10:1 ratio, indicating the presence of a residual population of macrophages that are refractory to the parasite. Since a 50% infectivity was required for our bioassay of the inhibition of entry, all further experiments were performed at parasite-to-macrophage ratios of 2:1 for amastigotes obtained from BALB/c mice and 10:1 for amastigotes from SCID mice. The uptake of amastigotes from the different sources into the macrophage-like cell line J774A.1 was similar to that observed for BMDM.

To identify other receptors used for entry, we examined the role of complement receptors because the use of CR1 and CR3 in the uptake of promastigotes of *Leishmania* spp. has been demonstrated (4, 10, 11, 17–20, 30, 33, 35). A single report, by Blackwell et al. (4), described a reduction in the entry of amastigotes of *Leishmania donovani* when the CR3 was blocked. In addition to having surface-bound Ig, amastigotes also possess host complement components on their surface (Fig. 4). The presence of complement component C3 on the parasite surface was determined by an

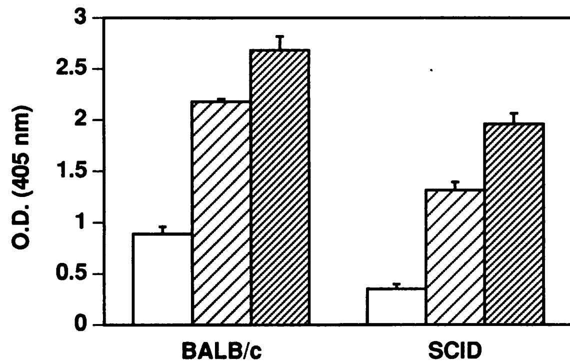

FIG. 4. Binding of anti-human C3 antibody to amastigotes from BALB/c and SCID mice. Shown are the mean optical density (O.D.) values from an ELISA. Prior to addition of anti-human C3, diluted GPS was either not added (□) or added at a dilution of 1:100 (▨) or 1:50 (▩) to each well containing parasites.

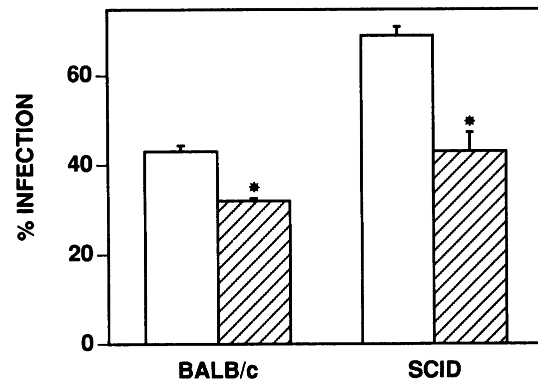

FIG. 5. Blocking of parasite entry into cells of the macrophage-like cell line J774A.1 in the presence of an anti-complement receptor MAb (either 25 μg of an MAb against CD44 per ml [□] or 25 μg of MAb M1/70 against the α-chain of CR3 per ml [▨] was added to the macrophages). The ratios of amastigotes to macrophages were 2:1 for amastigotes from BALB/c mice and 10:1 for those from SCID mice. The results are expressed as the mean percentage of entry of three replicates ± standard error. These data are representative of more than three identical experiments with similar results. *, significantly different (by analysis of variance; $P < 0.01$).

ELISA with rabbit anti-human C3 (Serotec; Cedarlane Labs, Hornby, Ontario, Canada) at a dilution of 1:2,000. The amount of complement components was much greater on amastigotes from BALB/c mice than those from SCID mice. Possibly, amastigotes from BALB/c mice may be better able to bind C3 because of the presence of Igs on their surface. Interestingly, the opsonization of amastigotes from SCID mice with guinea pig serum (GPS; the source of complement) resulted in a 68% increase in the level of infection of macrophages in vitro (data not shown). Heat inactivation of GPS abrogated this effect.

We examined the use of complement receptors by blocking the receptors with MAb M1/70 against the α-chain of the CR3. MAbs were produced from the cell line (ATCC) and purified from hybridoma supernatant by ammonium sulfate precipitation and S-200 gel chromatography. An MAb against the surface antigen PGP-1 (CD44), which bound to the surface of the macrophages, was used as a control. MAb M1/70 did not recognize the murine BMDM we used for our assays; consequently, the macrophage-like cells of line J774A.1, which are recognized by M1/70, were used for these assays. A dose-response curve indicated that 25 μg/ml gave maximal inhibition, and thus, this was the concentration used in the assays. MAb M1/70 was effective in reducing the amastigote entry, suggesting that the CR3 is utilized by the parasite to enter macrophages. The anti-CR3 MAb was equally effective in blocking the entry of amastigotes from the two sources into macrophages, despite the differential amastigote/macrophage ratios used for amastigotes from BALB/c and SCID mice (2:1 and 10:1, respectively; Fig. 5). We consistently found a significant reduction in entry, ranging from 25 to 35%. Similar results were obtained by using murine resident peritoneal macrophages which possess the epitope recognized by MAb M1/70. This degree of inhibition was not enhanced by increasing the concentration of the MAb, altering the time of preincubation of macrophages with the MAb or the incubation temperature, or by down-modulating the CR3 by adhering macrophages to M1/70-coated slides. There is some controversy as to whether M1/70 can modulate FcRs. Brown et al. (5) reported a decrease in phagocytosis of Ig-opsonized sheep erythrocytes by monocytes in the presence of M1/70, whereas Kimura and Griffin (15) did not observe inhibition of phagocytosis of Ig-opsonized cells by murine peritoneal macrophages. We observed a reduction in the entry of amastigotes from SCID mice, which, lacking surface-bound IgG, cannot bind to FcRs. Our results indicate that the CR3 was blocked by M1/70.

To examine the role of complement receptors further, we treated macrophages with the synthetic microtubule inhibitor nocodazole (NOC) and quantified the uptake of amastigotes (Table 2). Newman et al. (23) demonstrated a reduced ability of NOC-treated macrophages to phagocytose C3b- and C3bi-opsonized sheep erythrocytes, whereas phagocytosis of IgG-opsonized cells was unaffected. We found a significant reduction in the uptake of amastigotes from SCID mice into NOC-treated macrophages and only a slight reduction in the uptake of amastigotes from BALB/c mice (Table 2).

Since blocking of the FcR did not inhibit the entry of the parasite, it appears that amastigotes have the ability to switch the receptors they bind. To address this issue, we blocked the FcR and CR3 simultaneously. Interestingly, when macrophages were adhered to immune complexes and then treated with either MAb M1/70, NOC, or a combination of both, we found a significant reduction in the entry of amastigotes from either BALB/c or SCID mice (Table 3).

The importance of the MFR for parasite entry was deter-

TABLE 2. Percent infection with *L. major* amastigotes, of macrophages treated with the synthetic compound NOC[a]

NOC treatment (μg/ml)	% Macrophages infected with amastigotes from[b]:	
	BALB/c mice	SCID mice
0	64 ± 1	40 ± 2
2.5	52 ± 3	33 ± 1
5.0	53 ± 1	21 ± 2[c]

[a] Macrophages, adhered to glass slides, were treated for 30 min with NOC prior to exposure to amastigotes. Control wells received 1% dimethyl sulfoxide in DMEM which was used to dissolve the NOC.

[b] Values are representative of three experiments with similar results, expressed as means ± standard errors.

[c] Significantly different (by analysis of variance; $P < 0.01$).

TABLE 3. Percent infection with *L. major* amastigotes (obtained from different sources) of macrophages adhered to immune complexes[a]

Treatment	BALB/c mice		SCID mice	
	% Infection[b]	% Inhibition	% Infection[b]	% Inhibition
Irrelevant MAb	48 ± 1	0	63 ± 5	0
MAb M1/70	32 ± 3	38	44 ± 1	30
NOC	25 ± 2	50	32 ± 1	49
M1/70 + NOC	19 ± 2	60	19 ± 1	70

[a] Macrophages were adhered to immune complexes and then preincubated for 30 min with inhibitors prior to a 1-h exposure to amastigotes. MAb was at a concentration of 25 μg/ml, and NOC was at 5 μg/ml. Percent infection of macrophages adhered to immune complexes but in the absence of treatment was 46% ± 4% with amastigotes from BALB/c mice and 61% ± 2% with amastigotes from SCID mice.

[b] Values represent the means ± standard errors of four replicates per group.

mined by testing the ability of mannan (Sigma) to block the uptake of amastigotes. We found no inhibition in the uptake of amastigotes from either BALB/c or SCID mice when macrophages were treated with up to 10 mg of soluble mannan per ml. Similarly, the entry of amastigotes into macrophages adhered to mannan-coated glass slides (1 to 10 mg of mannan per ml) was not inhibited. Thus, the MFR does not appear to be used by the amastigote stage of *L. major*.

The receptor-mediated entry of intracellular pathogens into macrophages has received much attention in the last 10 years. Although differences are apparent between the organisms in the types of receptors used and the requirement for opsonization, it is evident that many intracellular pathogens use the MFR, FcR, and/or complement receptors for entry into macrophages. For example, the yeast stage of the fungal pathogen *Histoplasma capsulatum* uses the CR3/LFA-1/p150,95 family of adhesion molecules to enter human macrophages, the MFR and FcR are not involved, and serum opsonization is unnecessary for entry (6). Uptake of the bacterium *Legionella pneumophila* proceeds by CR1 and CR3; however, unlike *H. capsulatum*, *L. pneumophila* requires opsonization with C3b and C3bi for binding to occur (26). *Mycobacterium leprae* also requires C3 opsonization for uptake by the CR1, CR3, and CR4 (32). Both the MFR and CR3 are used in entry by *Mycobacterium avium* and *Mycobacterium tuberculosis*, and while the presence of serum increases the rate of uptake, unopsonized organisms also enter by binding these receptors (3, 31). An organism that uses the MFR but not the FcR or CR3 is *Pneumocystis carinii* (12).

Numerous studies have examined the relationship between *Leishmania* spp. and receptors for entry into their host cell, the macrophage. The emphasis of these studies has been on examining the promastigote, or the insect stage of the parasite, while the entry of the tissue stage, the amastigote, has not received attention. Yet, the disease leishmaniasis is caused by the proliferation of amastigotes within tissues of the mammalian host. Adapted for an intracellular existence, the amastigote bears little resemblance to the flagellated promastigote. It is therefore likely that these two distinct forms evolved different mechanisms for entry into host cells.

The present study provides evidence that promastigotes and amastigotes of *L. major* both share and have distinct receptors for entry into macrophages. The observed differences in the receptors used may reflect the altered environments to which the promastigotes and amastigotes are exposed. The promastigotes which initiate the infection are in an early inflammatory environment, whereas the tissue in which the amastigotes reside is an acute inflammatory environment. It is expected that the amastigotes are exposed to a variety of inflammatory molecules and host cells not encountered by promastigotes. This may have caused differential selection pressures for alternate mechanisms of entry of the two stages of *Leishmania* spp. into macrophages. Although opsonization with parasite-specific IgG enhanced the entry of promastigotes, the FcR is probably not used for entry of promastigotes in vivo (primary infection) since no parasite-specific antibody is present at the site of inoculation. This receptor, however, is probably very important for the entry of amastigotes in vivo and subsequent growth and development of the leishmanial lesion. In addition to the macrophage receptors tested in this study, it is likely that other as-yet-unidentified receptors may be involved in the entry of the amastigotes into macrophages. For example, we found that amastigotes isolated from SCID mice have no surface-bound Ig and very little complement bound to their surface, yet SCID mice are highly susceptible to infection with amastigotes of *L. major*, suggesting that the uptake of the parasites by SCID mouse macrophages may be regulated by different receptor-ligand interactions.

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