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THE MOUSE MACROPHAGE RECEPTOR FOR C3bi (CR3) IS A MAJOR MECHANISM IN THE PHAGOCYTOSIS OF LEISHMANIA PROMASTIGOTES[1]

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We examined the role of the macrophage receptor for C3bi, the CR3, in the phaocytosis of *Leishmania major* promastigotes and report that M1/70, a monoclonal antibody to the CR3, inhibited the binding of leishmania to macrophages both when the assays were performed in the presence of normal serum and in its absence. In serum, leishmania activate complement and fix C3. Fixation and subsequent cleavage to C3bi occurs rapidly, and by as early as 5 min both forms of the molecule can be identified on the parasites' surface. Complement fixation results in an enhanced phagocytosis of leishmania promastigotes by mouse macrophages. In the case of *L. major*, 63% of this serum-enhanced binding is inhibitable by M1/70.

Binding assays were also performed in the absence of serum with the use of thoroughly washed promastigotes. The addition of M1/70 inhibited binding under these conditions by 54%. Two other rat monoclonal antibodies directed against different antigens on the macrophage plasma membrane did not inhibit binding. M1/70 did not inhibit the binding of promastigotes to rat bone marrow cells, nor did it inhibit IgG-SRBC binding to mouse peritoneal macrophages. These data indicate that the inhibition observed in the presence of M1/70 was specific for the CR3 and that the macrophage receptor for C3bi plays a major role in the phagocytosis of *Leishmania major* promastigotes, even in the absence of serum.

Leishmania are obligate intracellular protozoan parasites. The flagellated promastigote form is injected into the mammalian host from the sandfly. Promastigotes enter dermal macrophages and multiply as amastigotes, the form characteristic of the vertebrate host. We have shown (1) that promastigotes of all species activate complement, and as a result, are susceptible to serum-mediated lysis. It is therefore essential that promastigotes gain rapid access into macrophages before lysis can occur. Chang (2) described the interaction of promastigotes with macrophages at the ultrastructural level and attributed parasite uptake to an active phagocytic process. Klempner and colleagues (3), using plasma membrane vesicles, suggested that promastigote binding occurred by a mechanism that was both specific and saturable. We have noted the importance of serum factors in this interaction (1). In this paper we present evidence that indicates that the enhanced binding of leishmania to macrophages in the presence of serum is due primarily to C3 fixation by the parasite and that the majority of serum-dependent binding occurs via the macrophage receptor for C3bi (CR3).[2] We also extend these observations to show that even in the absence of serum a considerable portion of leishmania uptake depends on the macrophage CR3. Although complement activation by promastigotes can lead to lysis, these data suggest that promastigotes may also take advantage of complement activation by rapidly converting C3b to C3bi and using this as a means to enter phagocytic cells via their complement receptors.

MATERIALS AND METHODS

Parasites. The National Institutes of Health S-strain of *Leishmania major* (formerly called *L. tropica major*) was originally isolated from a patient with a cutaneous ulcer in West Africa. It was provided by Dr. D. Wyler, Tufts University Medical Center (4).

Macrophages. Resident macrophages were washed from the peritoneal cavity of CDF_1 mice (Charles River Breeding Laboratories, Wilmington, MA) as described (5). Cells were washed and incubated overnight in DMEM supplemented with 10% FCS. Before the addition of leishmania, cells were washed three times with serum-free phagocytosis buffer (PB), which contains 1% BSA and equal parts of DMEM and TC199 buffered with 25 mM HEPES.

Leishmania binding assay. Tritium-labeled parasites in phagocytosis buffer were added to the washed macrophage monolayer for 45 min at 37°C. Parasites were labeled by resuspending them to a concentration of 8×10^7 organisms/ml in Schneider's complete medium containing 8 μCi/ml of [^{3}H]uracil (specific activity = 20 Ci/mM; New England Nuclear, Boston, MA) for 3 hr. Labeled promastigotes were washed three times and resuspended to 2×10^7 parasites/ml in PB. Parasites were then added to the monolayer, and the volume was corrected to 400 μl. As the promastigotes remained in suspension for the 45 min incubation, the amount of parasites added was expressed as parasites/ml in Figures 2, 3, and 4. For experiments involving serum, 16 μl of normal guinea pig serum (NGPS) were added, giving a final serum concentration of 4%. After the incubation, monolayers were thoroughly washed and processed for scintillation counting by adding 300 μl of 0.5% Triton X-100 per well. The total number of parasites per coverslip was determined by preparing a standard curve by using samples of parasites from the original labeled parasite suspension as described (1). The number of macrophages per coverslip was determined by counting the number of macrophages present in five randomly chosen areas of a calibrated grid eyepiece and normalizing to total coverslip area. Coverslips contained an average of approximately 1.2×10^5 macrophages. The mean number of promastigotes per macrophage was calculated by using the relationship leishmania/macrophage = total parasites per

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[2] Abbreviations used in this paper: CR3, the receptor that recognizes C3bi; NGPS, normal guinea pig serum; C8D, human serum deficient in the eighth component of complement; PB, phagocytosis buffer containing 1% BSA and equal parts of Dulbecco's minimal essential medium and tissue culture 199 medium buffered with 25 mM HEPES; IgG-SRBC, sheep red blood cells sensitized with IgG; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

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coverslip/total macrophages/coverslip. The total incorporation of radioactive uracil by parasites varied from day to day between 7×10^{-3} and 5×10^{-2} cpm/parasite. Total counts per coverslip averaged 4000 in the absence of serum. All the data listed in this paper were derived from radiobinding assays. In every case, however, light microscopy was performed in parallel to qualitatively assess the effect of the various treatments. The data reported reflect total macrophage-associated radioactivity. By light microscopy approximately 70% of the parasites have been internalized at the end of the 45-min incubation period. There was never an instance when an experimental treatment reported in this paper effected preferentially either binding or internalization.

Immunofluorescence. To determine the form of the C3 molecule on the parasite, immunofluorescence was performed by using rat monoclonal antibodies to human C3 (6), generously provided by Dr. P. J. Lachmann, MRC Cambridge, England. The clone 4 monoclonal antibody recognizes the C3c portion of the molecule and reacts with both C3b and C3bi. It was used as ascites at a dilution of 1/533. The clone 9 monoclonal antibody recognizes a "neoantigen" uncovered on C3bi but does not react with C3b. It was also used as ascites at a dilution of 1/333. Parasites were sensitized with 15% C8-deficient serum (C8D) for either 5 or 15 min at 35°C. Sensitized organisms were washed and fixed in 0.5% buffered formalin at 4°C for 2 hr. Monoclonal antibodies were added for 1 hr at 4°C, followed by a biotin-conjugated antibody to rat IgG and fluorescein isothiocyanate (FITC)-conjugated avidin, each for 1 hr at 4°C, with two washes between. Parasites sensitized with heat-inactivated C8D serum (56°C, 30 min) or normal serum at 4°C in the presence of EDTA did not fluoresce.

Monoclonal antibodies. M1/70 is a rat monoclonal antibody that reacts with the mouse macrophage receptor for C3bi, the CR3 (7). It was originally raised by Dr. T. Springer, Harvard Medical School (8). Hybridomas producing M1/70 were obtained from American Type Culture Collection and grown in DMEM supplemented 3 mM glutamine and 12% FCS. Culture supernatants from these cells were pooled, then precipitated with ammonium sulfate. The mixture was dialyzed for 48 hr at 4°C against borate-buffered saline at pH 8.0 and frozen in small fractions at a protein concentration of approximately 4 mg/ml. The antibody was diluted 1/100 in PB before use. The dilution of 1/100 was selected as the concentration of antibody that maximally inhibited the binding of C3bi-coated rabbit red blood cells but did not significantly diminish the binding of IgG-coated sheep red blood cells (IgG-SRBC). In the presence of M1/70, the percentage of macrophages binding three or more rabbit red blood cells decreased from 96% to 7%, whereas sheep red blood cell binding was reduced by less than 8%.

2.4G2 is a rat monoclonal antibody to the trypsin-resistant receptor on mouse macrophages for the Fc portion of immunoglobulin, the FCR 2 (9). It was originally raised by Dr J. Unkeless, Rockefeller University. The antibody, which was obtained from ascites, was precipitated with 45% ammonium sulfate and purified on a DE52 column. It was used in a final concentration of 10 μg/ml in PB. 2D2C is a rat monoclonal antibody to a major mouse macrophage plasma membrane protein (10). It was also raised by Dr. J. Unkeless and was used in the phagocytosis assay at a concentration of 20 μg/ml as described for 2.4G2. Both antibodies were generously provided by Dr. E. Pure and Dr. J. Unkeless, Rockefeller University. In our hands, approximately 1 μg/ml of 2.4G2 and 2 μg/ml of 2D2C were saturating, as determined by an immunofluorescence assay to detect macrophage-bound immunoglobulin. By this criteria, each was used in a 10-fold excess.

RESULTS

Serum sensitization results in C3b and C3bi on the surface of the parasite. When parasites are exposed to fresh serum they fix C3. We performed immunofluorescence microscopy to determine the form of the molecule on the surface of the parasite and the amount of time required for the conversion from C3b to C3bi (Fig. 1). C3b was identified by the clone 4 monoclonal antibody to C3b, raised by Lachmann (6), which recognizes the C3c portion of the molecule. C3bi was recognized with the clone 9 monoclonal antibody, which reacts with a neoantigen expressed on C3bi but does not react with C3b. After an incubation in serum of as little as 5 min, both C3b and C3bi were identified on the surface of promastigotes, indicating a rapid conversion from the functional

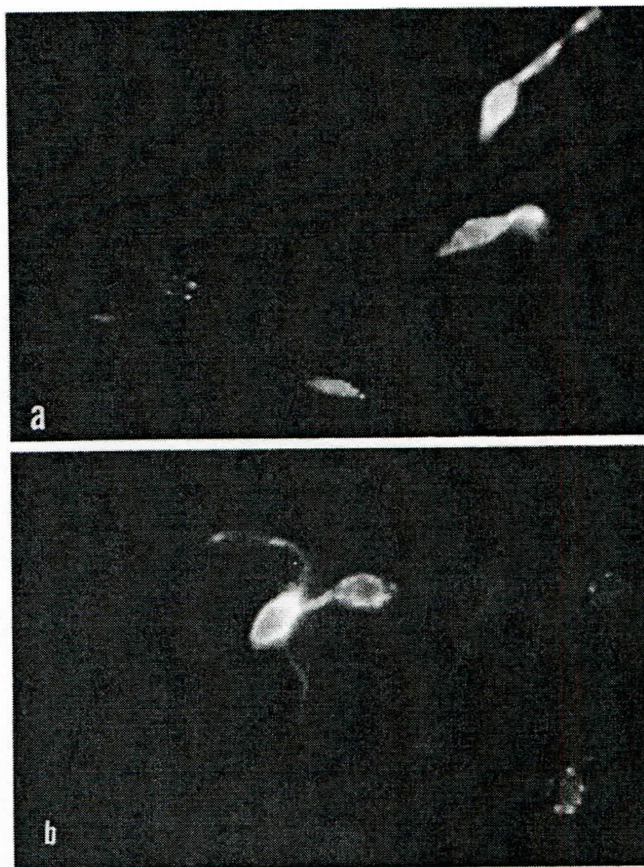

Figure 1. Indirect immunofluorescence staining of *L. major* promastigotes by using monoclonal antibodies to C3. Promastigotes were sensitized with 15% C3D serum for 5 min at 37°C, fixed with 0.5% formalin, and stained with either clone 4 monoclonal antibody, which reacts with C3b (*a*), or clone 9, which reacts with C3bi (*b*).

C3b to its inactive form, C3bi. The intensity of fluorescence with each monoclonal antibody reached a plateau by 15 min. Parasites incubated in heat-inactivated serum (56°C, 30 min) or in the presence of EDTA at 4°C did not fluoresce.

M1/70 inhibits the serum-dependent binding of promastigotes to macrophages. We showed previously that when macrophages are exposed to leishmania promastigotes in the presence of normal serum, they exhibit up to a fivefold enhancement of their ability to bind promastigotes relative to the amount of binding in the absence of serum (1). In this paper we attribute serum-enhanced binding to the macrophage receptor for C3bi, the CR3. In 4% NGPS, macrophages bind an average of 3.5 parasites per macrophage. This binding decreases to 1.8 parasites per macrophage in the presence of a 1/100 dilution of M1/70, a decrease of 49% (Table I). To investigate the serum-dependent mechanisms, phagocytosis assays were performed in EGTA[2]/Mg, conditions in which complement receptors remain functional but the residual serum-independent mechanisms do not (1). The amount of binding under these conditions was parasite dose dependent (Fig. 2). In the presence of M1/70, serum-dependent binding was decreased by approximately 60% at each concentration of parasites added, indicating the involvement of the CR3 in the serum-dependent binding of *L. major* promastigotes.

When promastigotes are exposed to 15% C8D serum

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TABLE I

The inhibition by M1/70 of L. major promastigotes binding to mouse macrophages[a]

Experiment	Treatment	(n)	Control[b]	M1/70[b]	% I[c]
Total binding	4% NGPS	(5)	3.45 ± 0.84	1.85 ± 0.53	49 ± 6
Serum-dependent binding	NGPS/EGTA[d]	(3)	2.05 ± 0.09	0.75 ± 0.12	63 ± 4
Serum-independent binding	PB	(7)	1.46 ± 0.29	0.71 ± 0.19	54 ± 3
CR3 binding	Factor 1/EGTA	(3)	1.81 ± 0.04	0.43 ± 0.04	76 ± 3

[a] Fifty microliters of 2×10^7 parasites/ml in phagocytosis buffer were added to 1.2×10^5 macrophages on coverslips for 45 min at 35°C.
[b] Mean ± SE.
[c] Mean percent inhibition of each individual determination.
[d] EGTA (5 mM) with 3 mM MgCl.

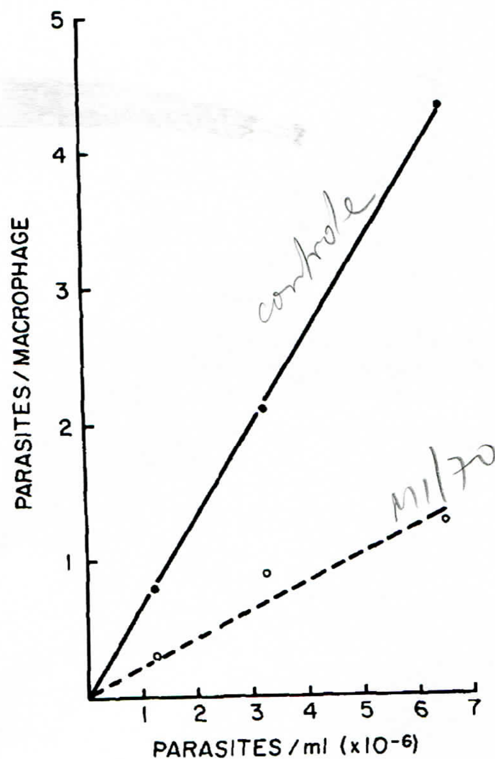

Figure 2. The inhibition of serum-dependent binding of radiolabeled *L. major* promastigotes to mouse macrophages by M1/70. Promastigotes were added to macrophage monolayers in the presence of 4% NGPS and 5 mM EGTA with 3 mM MgCl. Controls (*closed circles*) also received buffer, whereas the experimental group (*open circles*) received M1/70. The ordinate designates the average number of parasites per macrophage as described in *Materials and Methods*. The abscissa indicates the three doses of parasites added, expressed as the number of parasites per milliliter in the final volume.

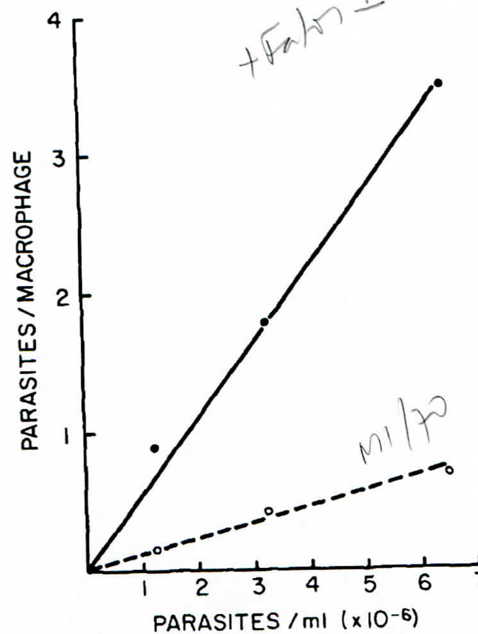

Figure 3. The inhibition of binding of C3bi-coated promastigotes to mouse macrophages by M1/70. Promastigotes of *L. major* were exposed to 15% C8D serum for 15 min and 70 U of Factor I for 30 min. They were then washed and added to the monolayer in the presence of EGTA/Mg for 45 min. Controls (*closed circles*) received buffer, whereas the experimental group (*open circles*) received M1/70.

for 15 min, and then partially purified factor I (Cordis, Miami, FL) for an additional 30 min, they become predominantly coated with C3bi rather than C3. The binding of washed C3bi parasites in the presence of M1/70 culture supernatants was reduced from 1.8 parasites/macrophage to 0.43 parasites/macrophage, a decrease of 76%. The same degree of inhibition was observed at each of the three concentrations of parasites tested (Fig. 3).

M1/70 inhibits the serum-independent binding of promastigotes to macrophages. In the absence of serum, macrophages bind an average of 1.5 promastigotes of *L. major* per cell. This binding decreases to 0.7 in the presence of M1/70, a decrease of 54%. The amount of inhibition remains relatively constant over the three doses of parasites added (Fig. 4). We performed controls to show that this inhibition was due only to the presence of an antibody to the CR3. Two other rat monoclonal antibodies that are specific for the macrophage plasma membrane but do not recognize the CR3 do not effect leishmania binding when added in excess of 10 times saturation (Table II). Three other controls were performed to rule out a nonspecific inhibitory effect. The binding of *L. major* promastigotes to rat bone marrow cells (4.4 parasites/macrophage) decreased by less than 8% in the presence of M1/70 (4.1 parasites/macrophage). Further, 86% of macrophages in the presence of M1/70 retain their ability to bind three or more IgG-SRBC. The binding of latex beads was also unaffected by the presence of M1/70 (data not shown).

DISCUSSION

In this paper we examine the involvement of the macrophage receptor for C3bi (CR3) in the phagocytosis of leishmania promastigotes. We report that the CR3 is a major mechanism by which promastigotes enter macrophages, and that this receptor is involved in leishmania phagocytosis both when the assays are performed in the presence of serum and under serum-free conditions.

In the presence of fresh normal serum, macrophage binding of leishmania increases by as much as fivefold, depending on the species tested (1). Serum sensitization results in deposition of C3b on the parasites' surface, with a rapid conversion to C3bi. At no time were we able to identify C3b on the parasite without C3bi being present. It is this rapid fixation and conversion of C3 to C3bi

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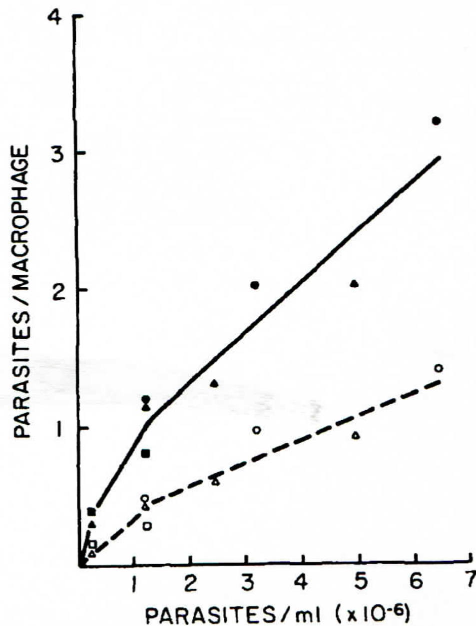

Figure 4. The inhibition of the serum-independent binding of *L. major* promastigotes to mouse macrophages by M1/70. Washed parasites in phagocytosis buffer were added to the monolayer for 45 min at 37°C. Controls (*closed circles*) received buffer, whereas the experimental group (*open circles*) received M1/70.

TABLE II

The inhibition of L. major binding to mouse macrophages by rat monoclonal antibodies

Treatment	Binding[a]	Percent Control[b]	(n)[c]
Control	1.45	—	—
M1/70	0.71	50 ± 5	(3)
2.4G2	1.68	109 ± 6	(6)
2D2C	1.29	94 ± 6	(7)

[a] Data are the average number of parasites/macrophage and were taken from one representative experiment.
[b] Mean ± SE.
[c] The number of individual determinants (n) refers to the *Percent Control* column, for which three experiments were performed at two different parasite concentrations.

that is responsible for the majority of serum-dependent *L. major* binding. Inhibition assays performed with M1/70, a monoclonal antibody to the CR3, indicate that when macrophages are cultivated in the presence of normal serum, approximately one-half of the total binding of promastigotes is attributable to the CR3. If we perform the assay in the absence of calcium, more than 60% of the serum-dependent binding is inhibited by blocking the CR3. The 40% residual binding not inhibitable by M1/70 undoubtedly represents in part promastigotes that have been bound by the CR1, the macrophage receptor for C3b. We also recognize the possibility that still other mechanisms may contribute to this serum-dependent enhancement of binding. In fact, we have some experimental evidence to indicate that his may be so: Binding of *L. major* promastigotes increases nearly twofold in the presence of high concentrations (20%) of FCS (unpublished data). And *L. major* binding in the continuous presence of serum is greater than when parasites are presensitized with serum and washed before their addition to the monolayer. We also add that the serum-dependent binding and inhibition studies reported here were performed with *L. major* and may not quantitatively reflect the effect of serum on every species of leishmania.

M1/70 also inhibits more than one-half the amount of promastigote binding that occurs in the *absence* of serum. This inhibition of serum-independent binding to macrophages appears to be specific for the CR3. Other rat monoclonal antibodies directed against different antigens on the macrophage plasma membrane do not inhibit leishmania binding. Further, inhibition does not occur when M1/70 is added to rat bone marrow cells, whose receptor for C3bi is not recognized by M1/70, arguing against a nonspecific inhibitory effert. We can only speculate on a mechanism to explain the inhibition of serum-independent binding by M1/70. Ezekowitz et al. (11) have proposed that the CR3 may be involved in the binding of zymosan, another alternative complement pathway activator. They attribute zymosan uptake to a local opsonization by macrophages, cells that have been shown to synthesize and secrete all the early alternative pathway proteins (12). Ross and colleagues (13), in contrast, have shown that the CR3 has lactin-like activity, suggesting the possibility that the macrophage recognizes some specific glycoprotein on the surface of *L. major* promastigotes. An alternative explanation would be that the antibody is reacting with a distinct receptor for leishmania that has an antigenic determinant common to the CR3 as well. A closely related family of three plasma membrane proteins, for example, each possessing the 94,000-dalton subunit found in the CR3, has been identified (14).

This paper extends our previous observation concerning the conditions under which leishmania bind to macrophages, in the presence or absence of serum (1). We now show that the serum-dependent binding of *L. major* to mouse macrophages is predominantly due to C3bi on the surface of the parasite and occurs primarily via the macrophage CR3. We show that a substantial portion of binding under serum-free conditions can also be inhibited by M1/70. The data suggest that complement activation may be a mechanism preserved by leishmania promastigotes to facilitate their entry into phagocytic cells.

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