MACROPHAGE COMPLEMENT AND LECTIN-LIKE RECEPTORS BIND *LEISHMANIA* IN THE ABSENCE OF SERUM

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Intracellular protozoa, *Leishmania* spp, *Trypanosoma cruzi*, and *Toxoplasma gondii*, have as a pathogenic characteristic a particular tropism for host mononuclear phagocytes. Successful invasion of host macrophages (Mϕ)[1] by *Leishmania* promastigotes requires an initial interaction at the cell surface that has not been defined. In the presence of serum, enhanced binding of *Leishmania* promastigotes to murine resident peritoneal Mϕ (RPM) is mediated by complement (1). In the absence of serum, the lectin-like Mϕ-specific mannosyl/fucosyl receptor (MFR) may play a role (2), consistent with studies (3, 4) demonstrating mannose-rich glycoconjugates on the surface of *L. donovani* promastigotes. We report an additional role for the type three complement receptor (CR3) in serum-independent binding of *L. donovani* to RPM. Modulation experiments in which either CR3 or MFR are rendered inaccessible demonstrate that both receptors must be present on the segment of Mϕ membrane to which the parasite binds. The combined function of these two distinct Mϕ receptors may represent a general mechanism for the entry of pathogenic protozoa into mononuclear phagocytes.

Materials and Methods

Mice. C57BL/10ScSn mice were from OLAC(1976) Ltd., Bicester, Oxon, or bred at the London School of Hygiene and Tropical Medicine and males or females used between 6 and 12 wk.

Reagents. The rat anti–mouse CR3 monoclonal antibody (mAb) M1/70 (anti-Mac-1) (5) was used at saturation (6). Fab fragments of M1/70 and rat anti–mouse mAb 2.4G2, which binds to Mϕ IgG2b/IgG1 Fc receptors (7), were kind gifts from Dr. T. Springer, Harvard Medical School, Boston and Dr. J. Unkeless, The Rockefeller University, New York. F4/80, a rat antibody (Ab) specific for mature mouse Mϕ (8), was used as concentrated hybridoma supernatant. Anti-C3 Fab, prepared from a rabbit anti–human C3 that crossreacts with mouse C3, was used at a concentration (200 μg/ml) known to

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CR3, complement receptor type 3; HINMS, heat-inactivated NMS; mAb, monoclonal antibody; MFR, mannosyl/fucosyl receptor; Mϕ, macrophage; NMS, normal mouse serum; RPM, resident peritoneal Mϕ.

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block all lymphocyte CR1 and CR2 rosetting (9). The nucleophile, sodium salicyl hydroxamate, was prepared as a 100 mM solution in 100 mM NaOH and diluted in culture medium to 1 mM. Mannan (M7504) and ribonuclease B (type XIIB) (both from Sigma Chemical Co., St. Louis, MO) were used as MFR inhibitors at 1 mg/ml.

Parasites. Details of the isolation of *L. donovani* amastigotes from hamster spleen, and of the transformation and culture of promastigotes, appear elsewhere (2). Promastigotes were used between days 3 and 7 of the 3rd to 17th subcultures, as indicated.

Mouse Mϕ. RPM were plated on washed, 13-mm glass coverslips (2) or on M1/70-, M1/70-Fab-, mannan-, or 2.4G2-Fab-coated coverslips prepared by the method of Michl et al. (10). The ability of Mϕ on coated coverslips to rosette iC3b-coated erythrocytes was assessed microscopically. Assays of ^{125}I-mannose-bovine serum albumin uptake or M1/70 binding by Mϕ on mannan- or M1/70-Fab-coated coverslips have been described (6).

Parasite/Mϕ Binding. In each experiment, 5×10^6 promastigotes or 2.5×10^6 amastigotes were added to two or four coverslips, which were then washed and examined after 20 min. Mϕ were scored individually (300–500 per coverslip) for attached and ingested parasites from Giemsa-stained fixed preparations (2). Mϕ were preincubated with soluble inhibitors for 15 min before and during the parasite incubation. Parasite binding to Mϕ was examined in the absence of serum or with 8–12% normal or heat-inactivated (56°C, 30 min) mouse serum (NMS or HINMS).

Alternative Pathway Activation. Consumption of complement in fresh human serum by promastigotes, amastigotes, and zymosan was compared (per parasite or particle) by a two-stage assay (11). Alternative pathway activation was also measured for mannan (6.25 mg/ml), parasites in the presence of mannan (6.25 mg/ml), and mannan-coated coverslips.

Results and Discussion

The role of locally secreted complement proteins in mediating binding to Mϕ CR3 has already been established for the yeast wall product zymosan (6). In initial experiments we examined the effects of the monoclonal anti-CR3 Ab M1/70 on serum-independent binding and ingestion of *L. donovani* promastigotes and amastigotes by RPM. Fig. 1*A* shows one experiment representative of 15 in which binding efficiency for control Mϕ was 30–260 parasites per 100 cells in 20 min, depending on the promastigote subculture used, and for which 53–88% inhibition of promastigote binding and ingestion was observed in the continuous presence of soluble M1/70. In control experiments, similar inhibition of promastigote binding was obtained after preincubation alone with M1/70. The unrelated Ab F4/80 caused no inhibition of promastigote binding. It is unlikely, therefore, that the inhibition observed with M1/70 was simply due to steric hindrance. In four parallel experiments with amastigotes, binding efficiency in control Mϕ was 130–320 parasites per 100 cells in 20 min, but only 14–43% M1/70 inhibition was observed. This difference in ability to block binding of pro- and amastigotes with M1/70 correlated with their relative abilities to activate the alternative pathway (Fig. 1*B*) and was mimicked by the use of the Fab fragment of anti-C3 Ab or the nucleophile, sodium salicyl hydroxamate (Fig. 1*A*). Sodium salicyl hydroxamate is a potent inhibitor of the covalent binding of activated C3 to the activator surface (12), providing strong evidence that cleaved C3, in the degraded iC3b form, on the surface of promastigotes mediates binding to CR3. Under similar assay conditions we have recently shown by sodium dodecyl sulfate–polyacrylamide gel analysis that Mϕ-derived cleaved C3 (iC3b) can be deposited on zymosan in the absence of exogenous complement (6 and Ezekowitz and Sim, unpublished results).

The present results establish a role for CR3 in the serum-independent binding

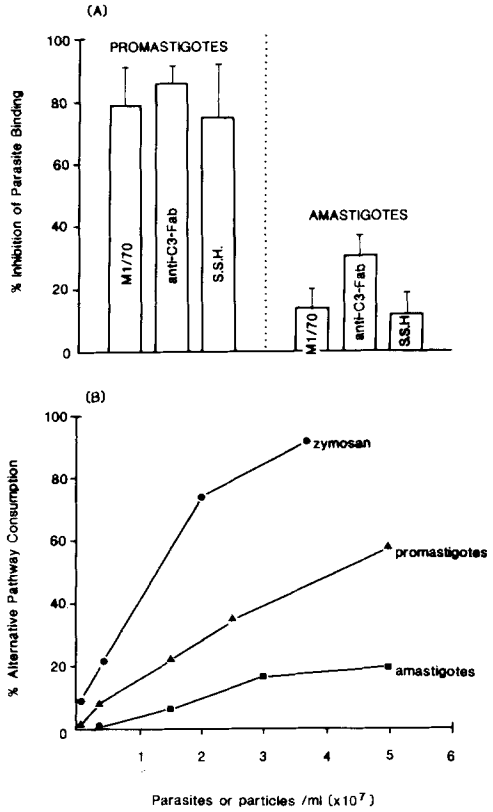

FIGURE 1. (*A*) One experiment in which the effect of blocking Mϕ CR3 activity using soluble M1/70 is compared with inhibition due to an anti-C3 Fab antiserum and the nucleophile, sodium salicyl hydroxamate (*S.S.H.*) for both promastigotes (day 7, subculture 3) and amastigotes of *L. donovani* examined on the same day. In this experiment, parasite binding in the absence of serum was 45 ± 8 promastigotes and 264 ± 22 amastigotes per 100 cells per 20 min for control Mϕ. The graph shows percent inhibition of parasite binding calculated for the 16 possible individual coverslip comparisons ($\bar{x}$ ± SD) made between quadruplicate treated and control coverslips. (*B*) The ability of promastigotes (day 4, subculture 8) and amastigotes to consume the alternative pathway is compared with the known activator zymosan.

of locally iC3b-opsonized promastigotes to the Mϕ surface. In view of our earlier observation (2) of MFR-mediated promastigote binding to RPM, we have determined the relative roles of these two receptors in parasite binding. A role for the MFR in uptake of zymosan has been demonstrated (13) and, in different Mϕ populations, additive effects of receptor blocking with mannan plus M1/70 were observed (6). In the present experiments (Fig. 2), the use of mannan or ribonuclease B as soluble inhibitors of MFR-mediated binding resulted in similar inhibition to that with M1/70: promastigotes, 50–80%, amastigotes, ~30%. In no case was there any evidence for an additive effect of blocking the two receptors simultaneously. Mannan (6.25 mg/ml) did not itself consume alternative pathway components or inhibit the ability of the parasite to activate the alternative pathway. It was unlikely, therefore, that the effects of mannan or ribonuclease B were due to interference with or inhibition of CR3-mediated binding of the

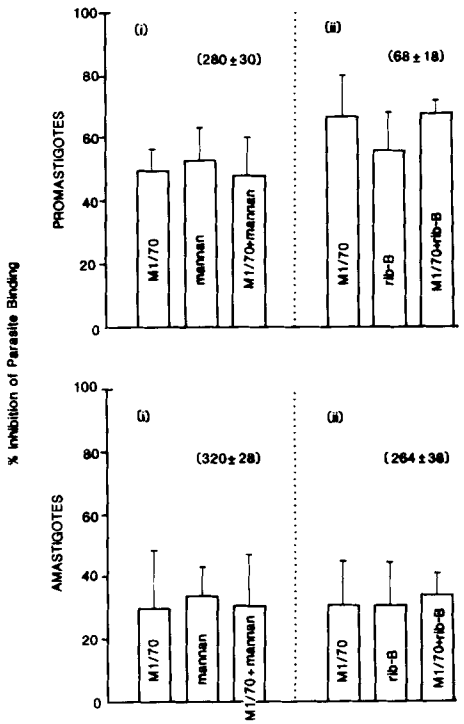

FIGURE 2. Effects of soluble inhibitors on CR3- and MFR-mediated uptake of *L. donovani* promastigotes (days 5 and 6, subcultures 10 and 6) and amastigotes by RPM. Results show percent inhibition of parasite binding in independent experiments examining (*i*) M1/70, mannan, or M1/70 + mannan, and (*ii*) M1/70, ribonuclease B (*rib-B*), or M1/70 + ribonuclease B. Numbers in brackets are the $\bar{x} \pm$ SD parasites per 100 cells per 20 min for control Mϕ.

parasite. Rather, it appeared that each receptor played an equivalent role in binding and ingestion of the parasite.

One explanation for the link between CR3 and MFR could be that they act sequentially, one receptor mediating attachment and the other promoting internalization. Our method of scoring parasites associated with Mϕ did not always allow us to distinguish these two functions. To clarify the roles of the two receptors, we performed additional experiments in which CR3 and MFR were selectively modulated onto M1/70-Fab- or mannan-coated coverslips. Mϕ plated on M1/70-Fab coverslips were unable to rosette iC3b-coated erythrocytes; specific binding of M1/70 was <10% compared with Mϕ plated directly onto glass (12,133 ± 416 cpm ^{125}I-labeled second stage Ab per coverslip). Specific uptake of ^{125}I-mannose-BSA was equivalent to that of control Mϕ (6,980 ± 198 cpm/coverslip during 20 min at 37°C). In the reciprocal experiment with cells plated on mannan-coated coverslips, iC3b-erythrocyte rosetting and binding of M1/70 (12,600 ± 1,414 cpm/coverslip) was equivalent to that of control Mϕ, but uptake of ^{125}I-mannose-BSA was reduced by 71%. These experiments established that CR3 and MFR are distinct receptors whose function can be modulated independently. In experiments with parasites (Fig. 3), equivalent reduction in binding and ingestion by Mϕ was observed after modulation of either CR3 or MFR:

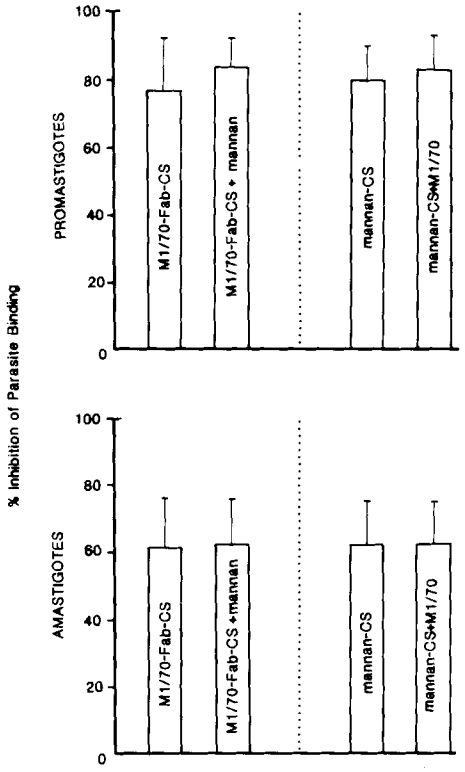

FIGURE 3. The effect of modulation of CR3 and MFR onto M1/70-Fab- and mannan-coated coverslips (*CS*) on serum-independent binding of *L. donovani* promastigotes (days 3–7, subcultures 5–17) and amastigotes by RPM. The graph shows percent inhibition ($\bar{x} \pm$ SD) of total parasite binding (attached plus ingested) over five independent experiments for Mϕ on coated coverslips with or without the addition of M1/70 or mannan (1 mg/ml) as soluble inhibitors. Parasite binding by control Mϕ was 202–265 promastigotes and 300–340 amastigotes per 100 Mϕ per 20 min.

promastigotes, ~80%, amastigotes, ~60%. The addition of soluble inhibitors did not, in either case, enhance the inhibition. In control experiments, no inhibition of parasite binding was observed for Mϕ where IgG2b/IgG1 Fc receptors were modulated onto 2.4G2-Fab-coated coverslips, and mannan-coated coverslips did not activate the alternative pathway.

These experiments demonstrate that, under serum-free conditions, both CR3 and MFR are required to promote attachment and ingestion of the parasite. Neither receptor alone is capable of performing even the lesser function of parasite attachment. Hence, an hypothesis based on sequential action of the two receptors is precluded, since at least one of them would be expected to bind the parasite. Nor does it appear that the remote signalling mechanisms described (14) for the combined action of fibronectin and complement receptors are operating. In that system, stimulation of the basal surface of monocyte plasma membranes by substrate-bound fibronectin was sufficient to activate complement receptors on the remote apical surface of the cell. In our studies, interaction of either CR3 or MFR with its respective surface-bound substrate (M1/70-Fab or

mannan) was not sufficient to promote attachment and ingestion by the receptor accessible to the parasite on the upper surface of the Mϕ. Apparently, both receptors must be present on the segment of membrane to which the parasite binds. This suggests some form of conformational co-recognition mechanism such as might occur if the same glycoconjugate molecule on the parasite surface were involved both in activating complement and in binding to MFR. Although earlier studies (3, 4) have shown mannose-rich glycoconjugates on the parasite surface, the covalent binding of activated C3 may be required to cause a necessary conformational change in the molecule, exposing the sugar residues that bind to MFR. As the MFR binds to this portion of the molecule, it may, in turn, promote maximal presentation of the binding site of iC3b to CR3. Further experiments are required to test these hypotheses.

As shown by others (1) fresh serum can enhance the binding of promastigotes of other species of *Leishmania* by Mϕ. In experiments with *L. donovani* we also observed dramatic increases in total promastigote binding to Mϕ in the presence of NMS. For example, control Mϕ bound or ingested 260 ± 8 promastigotes per 100 Mϕ during 20 min in the absence of serum, 1,095 ± 95 in the presence of 8% NMS, and 438 ± 78 in the presence of 8% HINMS. In the same experiment, 320 ± 28 amastigotes per 100 Mϕ during 20 min were bound or ingested in the absence of serum, 235 ± 71 with 8% NMS, and 184 ± 15 with 8% HINMS. Hence, for amastigotes there was no serum-induced enhancement of binding. In these experiments, inhibition of promastigote binding (Fig. 4) using soluble M1/70, anti-C3 Fab, or sodium salicyl hydroxamate, or using M1/70-coated coverslips, was higher (70–95%) than that observed with soluble mannan or mannan-coated coverslips (~35%). A larger proportion of the enhanced serum-dependent binding of promastigotes can therefore be accounted for by the activity of CR3, which in this case functions at least partially independently of the MFR.

In this study we have shown that Mϕ-derived complement components are

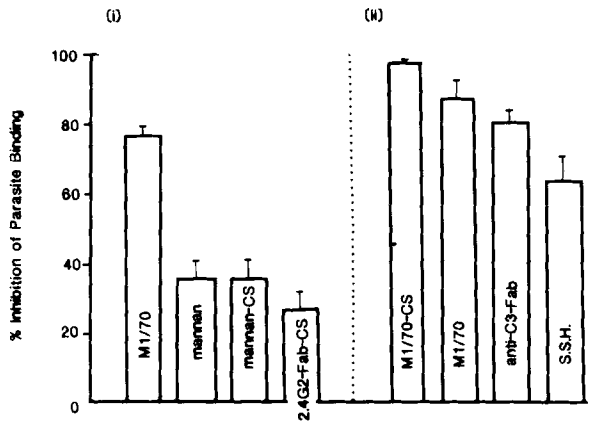

FIGURE 4. Effect of Mϕ receptor inhibition or modulation on serum-dependent binding of *L. donovani* promastigotes. The graph shows percent inhibition ($\bar{x}$ ± SD) of promastigote (day 7, subcultures 8 and 3) binding obtained in two independent experiments using (*i*) soluble M1/70, soluble mannan, mannan-coated coverslips (*CS*), or 2.4G2-Fab-coated coverslips, and (*ii*) M1/70-coated coverslips, soluble M1/70, anti-C3 Fab, and sodium salicyl hydroxamate (*S.S.H.*).

sufficient to mediate local opsonization of *Leishmania* and binding to CR3 that, in the absence of serum, requires the presence of an additional Mϕ receptor, MFR. Although the precise mechanism for the combined action of these two receptors is not understood, the constitutive ability of Mϕ to produce complement components may play an important role in recognition and uptake of this and many other parasites known to activate the alternative pathway.

Summary

We have examined the relative roles of the macrophage (Mϕ) plasma membrane receptor for the cleaved third complement component (iC3b, CR3) and of the mannosyl/fucosyl receptor (MFR) in binding and ingestion of *Leishmania donovani*. In the absence of exogenous complement, the binding and ingestion of promastigotes, which are good activators of the alternative complement pathway, were inhibited by the anti-CR3 monoclonal antibody M1/70, by the Fab portion of an anti-C3 antibody, or by the nucleophile, sodium salicyl hydroxamate, an inhibitor of C3 fixation. This provides strong evidence that Mϕ-derived, cleaved C3 (iC3b) present on the promastigote surface mediates binding to CR3. Equivalent inhibition of promastigote binding and ingestion was also observed using the soluble inhibitors of MFR activity, mannan or ribonuclease B. No additive effect for blocking the two Mϕ receptors simultaneously was observed. For amastigotes, which are poor activators of the alternative pathway, a lesser but nevertheless equivalent effect was observed for the three soluble inhibitors of CR3-mediated binding vs. the two soluble inhibitors of MFR-mediated binding. Modulation experiments in which either CR3 or MFR had been rendered inaccessible demonstrated that both receptors must be present on the segment of Mϕ membrane to which the parasite binds. The combined function of these two distinct Mϕ receptors may provide a general mechanism for recognition and ingestion of other pathogenic protozoa known to activate the alternative pathway.

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