MACROPHAGE TYPE 3 COMPLEMENT RECEPTORS
MEDIATE SERUM-INDEPENDENT BINDING OF
LEISHMANIA DONOVANI

Detection of Macrophage-derived Complement on the Parasite Surface
by Immunoelectron Microscopy

BY ANNE O. WOZENCRAFT,* GILLIAN SAYERS,† AND
JENEFER M. BLACKWELL*  
From the *Department of Tropical Hygiene and the §Wolfson Tropical Pathology Unit, London
School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom

In a recent study (1), we demonstrated a role for the macrophage (Mφ) type 3 complement receptor (CR3) in serum-independent binding of Leishmania donovani promastigotes to murine resident peritoneal Mφ (RPM). Earlier studies had also established a role for CR3 in serum-independent binding of the yeast wall product zymosan to murine and human Mφ (2) and to human neutrophils (3). In all three studies, binding of the parasite/particle was inhibited by mAbs (M1/70, Mo1, OKM1, MN-41, and anti-Leu-15) directed against the α-chain of CR3. In the neutrophil system it was argued that binding of zymosan to CR3 involved direct lectin-like recognition processes not mediated by complement, as binding was inhibited by an anti-CR3 mAb (OKM1) not specifically directed against the α-chain of CR3. In the neutrophil system it was postulated that complement proteins secreted by Mφ mediate local iC3b-opsonization of zymosan and promastigotes, both of which are good activators of the alternative complement pathway (1, 2). This was supported by three independent lines of evidence. First, binding of zymosan to Mφ was also reduced after treatment of Mφ with cyclohexamide, an inhibitor of protein synthesis (2). Second, the potent inhibition of promastigote binding to RPM using M1/70 was completely mimicked with Fab anti-C3 or the nucleophile sodium salicyl hydroxamate (1). The latter is a potent inhibitor of the covalent binding of activated C3 to the activator surface (4), thus providing strong evidence that cleaved C3, in the degraded form iC3b, on the surface of promastigotes mediates binding to CR3. Finally, under assay conditions identical to those used in binding assays, it was shown (2) by SDS polyacrylamide gel analysis that Mφ-derived C3 (iC3b) can be deposited on zymosan in the absence of exogenous complement. In this study, the evidence for local iC3b-oponization is extended by direct visualization of Mφ-derived C3 on the surface of L. donovani promastigotes using an anti-C3 antibody and a protein A–gold conjugate in electron microscope sections.

This work was supported by grants from the Medical Research Council and the Wellcome Trust. J. M. Blackwell is a Wellcome Trust Senior Lecturer.
Materials and Methods

*Mice.* Female C57BL/10ScSn mice from OLAC (1976) Ltd., Bicester, Oxon, United Kingdom, were used at 6-10 wk of age.

*Reagents.* The \( \gamma \) globulin fraction (not affinity purified) of rabbit anti-human C3 was kindly provided by Dr. R. Sim, Medical Research Council Immunochemistry Unit, Department of Biochemistry, Oxford University, Oxford, United Kingdom. This antibody crossreacts with mouse C3 and is the same as that used for preparation of the Fab anti-C3 which blocked *L. donovani* promastigote binding in the earlier study (1). Sodium salicyl hydroxamate was prepared as a 100 mM solution of salicyl hydroxamic acid in 100 mM NaOH and diluted in culture medium to 1 mM. Protein A-gold conjugate was used as a 1:100 dilution of stock protein A (18 \( \mu \)g/ml) labelled with 10-nm gold particles (E-Y Laboratories Inc., San Mateo, CA, generously provided by Dr. E. Handman, Walter and Eliza Hall Institute, Victoria, Australia).

*Media.* Bicarbonate-buffered (pH 7.3) medium 199 (m199) supplemented with 20 mM L-glutamine, 10 mM sodium pyruvate, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin was used for MO cultures. Where stated, this was further supplemented with 10% vol/vol heat-inactivated (56°C, 30 min) FCS (Gibco, Paisley, United Kingdom) or 10% freshly prepared autologous normal mouse serum (NMS).

*Parasites.* *L. donovani* (LV9) promastigotes were transformed from amastigotes and maintained by weekly passage as described previously (1). Promastigotes in stationary phase were harvested between days 6 and 10 of the third to tenth subcultures, washed three times in m199, and resuspended at \( 5 \times 10^6 \) parasites/ml.

*Mouse MO.* RPM were obtained by lavage (6) and plated onto 13-mm Lux Thermorx (Lab-Tek; Miles Laboratories, Inc., Naperville, IL) coverslips in Nunc tissue culture plates at \( 5 \times 10^5 \) large cells per coverslip. After adherence, MO were washed and incubated at 37°C in 5% CO\(_2\) and 95% air overnight in m199 with 10% heat-inactivated FCS.

*Detection of C3 Parasite Surface During Invasion.* MO cultures were washed thoroughly with m199 and pretreated for 30 min with 5 \( \mu \)g/ml cytochalasin B (Aldrich Chemicals Ltd., Gillingham, United Kingdom). This to reduce internalization of the parasites and to maximize visualization of C3 on the parasite surface. Coverslips were washed thoroughly with m199 and promastigotes were added at \( 5 \times 10^6 \) parasites/well. As a negative control, some coverslips were incubated with 1 mM sodium salicyl hydroxamate for 15 min before and during the parasite incubation. An exogenous source of complement components (10% NMS) was added with the parasites in positive control wells. Parasites were allowed to interact with MO for 20 min. Coverslips were then washed with m199 and the cells fixed with 1% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature. Coverslips were then incubated for 30 min at room temperature with 250 \( \mu \)l of 60 \( \mu \)g/ml \( \gamma \) globulin rabbit anti-human C3, washed thoroughly, and incubated for a further 45 min with 250 \( \mu \)l of 0.45 \( \mu \)g/ml protein A-gold conjugate. As an additional negative control, the anti-C3 antibody treatment was omitted on some coverslips. Cells were washed and fixed in 3% glutaraldehyde in 0.066 M cacodylate buffer (pH 7.4) for 60 min at 4°C.

*Electron Microscopy.* Coverslips were washed five times in 0.066 M cacodylate buffer over 12 h. After 60 min postfixation in 1% osmium tetroxide, cells were washed three times with buffer and dehydrated through a graded methanol series. Coverslips were dipped in propylene oxide, left overnight to infiltrate with Epon 812 (Araldite; EMscope Ltd., Ashford, United Kingdom), polymerized, sectioned, and stained with uranyl acetate and lead citrate. Sections were examined in a JEOL 100cX microscope at 80 kV.

Results and Discussion

In an earlier study (1) we concluded that serum-independent binding of *L. donovani* promastigotes to CR3 of murine RPM was mediated by locally secreted complement proteins. This conclusion was based on three independent but nevertheless indirect pieces of evidence, namely that binding could be inhibited by an antibody (M1/70) directed against the iC3b-binding site of CR3, by Fab...
anti-C3, or by the nucleophile sodium salicyl hydroxamate, which inhibits C3 fixation. In the present study, we provide direct visual evidence for local opsonization of L. donovani by Mφ-derived complement. In electron micrographs (Fig. 1, A and B), deposition of gold particles around stationary-phase promastigotes incubated with Mφ under serum-free conditions was achieved using specific antibodies directed against C3 followed by gold-labelled protein A. The amount of C3 deposited on the promastigote surface compares favorably with that observed (Fig. 1 C) when an exogenous (serum) source of C3 was supplied. In the latter case, however, severe damage to the promastigote accompanied C3 fixation, presumably due to availability of terminal components of the alternative complement pathway and formation of the membrane attack complex. This was not observed under serum-free conditions. In the serum-free system, no nonspecific binding of labelled protein A was detected if the anti-C3 antibody was omitted (Fig. 1 D). The presence of sodium salicyl hydroxamate abrogated promastigote binding to Mφ to the extent that none were found in association with Mφ despite extensive scanning of the ultrathin sections. This is consistent with our earlier observations (1) and provides further indirect evidence that it is covalently bound cleaved C3, in the degraded form iC3b, that mediates promastigote binding to CR3.

In our earlier study (1) we were unable to obtain a significant reduction of serum-free binding of the amastigote form of the L. donovani to murine RPM using the three soluble inhibitors M1/70, Fab anti-C3, or sodium salicyl hydroxamate. ~60% inhibition of amastigote binding was obtained, however, when
CR3 was modulated onto M1/70-Fab–coated coverslips, suggesting that their binding is at least partially mediated by CR3. In the present study, Mφ-derived C3 was observed on the amastigote surface (Fig. 2A) under serum-free conditions. However, since the anti-C3 antibody we used recognizes determinants common to native C3 and its breakdown products, we cannot be sure whether all or any of the bound C3 visualized by immunostaining is in the correct form (iC3b) to mediate binding to CR3. Other observations suggest that, although promastigotes and amastigotes of *Leishmania* subspecies share the predominant C3-binding glycoprotein gp63 (5, 6), the fate of C3 bound to the surface of the two forms of the parasite may be different. Gold deposition was not observed around amastigotes when the anti-C3 antibody was omitted (Fig. 2B), but was observed around amastigotes that bound to Mφ in the presence of sodium salicyl hydroxamate (Fig. 2C). This suggests that a proportion of the binding of C3 to the amastigote surface may be via noncovalent linkages. Addition of 10% NMS to the *Leishmania*/Mφ system also had dramatically different effects on amastigotes and promastigotes. For amastigotes, increased deposition of C3 occurred (Fig. 2D), but this did not mediate the enhanced binding (1) or damage observed with promastigotes. This is consistent with earlier observations that *Leishmania* promastigotes are damaged by incubation with normal human or guinea pig.
serum (7), whereas amastigotes are relatively resistant to lysis (8). This may, in turn, reflect the ability of amastigotes to block the complement cascade before formation of the membrane attack complex as observed for Trypanosoma brucei gambiense (9), or may indicate the presence of polysaccharide side chains, such as those on bacterial surfaces (10), which prevent the formation of macromolecular complexes by steric hindrance. Further work is required to ascertain the fate of bound C3 on promastigote vs. amastigote surfaces, and to determine whether such differences also relate to the differences in infectivity of logarithmic and stationary phase promastigotes (11), which also differ in their resistance to complement-mediated damage (12).

In conclusion, we have shown that Mø-derived complement proteins can be visualized on the surface of L. donovani promastigotes and amastigotes, thus providing the potential for local opsonization and recognition via CR3. Promastigotes appear to be dependent upon this system of local opsonization to gain entry into host Mø, but further work is required to determine whether amastigote binding, which is inhibitable by modulation of CR3 but not by the soluble inhibitors M1/70, Fab anti-C3, or sodium salicyl hydroxamate, involves direct lectin-like binding to CR3 (3) and/or other receptors. The extent to which the local opsonization system may be used by other microorganisms with C3-binding glycoproteins on their surface (e.g., T. cruzi [13]) and by other Mø populations will depend both on the ability of the different Mø subpopulations to secrete complement proteins, and on the fate of the C3 bound to the activator surface. It is clear, nevertheless, that whereas activation of complement pathways by bacteria and parasitic protozoa has conventionally been thought of as a process detrimental to the invading microorganism, the system described for Leishmania in this and our earlier paper (1) puts the parasite at a selective advantage in gaining access to its preferred host cell.

Summary

In this study, direct visual evidence for local opsonization of L. donovani by macrophage (Mø)-derived complement components was obtained using immunoelectron microscopy. C3 deposition was detected on the surface of both promastigotes and amastigotes after 20 min serum-free incubation with murine resident peritoneal Mø (RPM), followed by fixation and incubation first with specific antibody directed against C3 and then with gold-labelled protein A. Gold deposition was not observed around either form of the parasite if the anti-C3 antibody was omitted. For promastigotes, the degree of C3 deposition under serum-free conditions was comparable with that observed in the presence of an exogenous (serum) source of C3, but did not result in the same severe damage to the parasite as did the latter. Addition of sodium salicyl hydroxamate, which prevents covalent binding of C3 to activator surfaces, abrogates promastigote binding. Hence, although the anti-C3 antibody did not distinguish between native C3 and its breakdown product iC3b, these data support our earlier conclusion that promastigote binding to the CR3 of murine RPM is complement dependent. For amastigotes, gold deposition and binding to murine RPM were not eliminated by sodium salicyl hydroxamate. The presence of normal mouse serum resulted in increased gold deposition, but did not mediate either enhanced
binding to Mφ or damage to the amastigote. These data suggest that a proportion of C3 binding to the amastigote surface may be via noncovalent linkages, and that the C3 bound may not be in the correct form to mediate binding to CR3.

Received for publication 9 June 1986.

References

1. Blackwell, J. M., R. A. B. Ezekowitz, M. B. Roberts, J. Y. Channon, R. B. Sim, and S. Gordon. 1985. Macrophage complement and lectin-like receptors bind Leishmania in the absence of serum. J. Exp. Med. 162:324.

2. Ezekowitz, R. A. B., R. B. Sim, M. Hill, and S. Gordon. 1984. Local opsonization by secreted macrophage complement components. Role of receptors for complement in uptake of zymosan. J. Exp. Med. 159:244.

3. Ross, G. D., J. A. Cain, and P. J. Lachmann. 1985. Membrane complement receptor type three (CR3) has lectin-like properties analogous to bovine conglutanin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. J. Immunol. 134:3307.

4. Sim, R. B., T. M. Twose, D. S. Peterson, and E. Sim. 1981. The covalent-binding reaction of complement component C3. Biochem. J. 193:115.

5. Russell, D., and H. Wilhelm. 1986. Attachment of Leishmania promastigotes to macrophages is inhibited by Fab fragments directed against a parasite surface glycoprotein, gp63. J. Immunol. 136:2613.

6. Colomer-Gould, V., L. G. Qunitas, J. Keithly, and N. Nogueira. 1985. A common major surface antigen on amastigotes and promastigotes of Leishmania species. J. Exp. Med. 162:902.

7. Mosser, D. M., and P. J. Edelson. 1984. Activation of the alternative complement pathway by Leishmania promastigotes: parasite lysis and attachment to macrophages. J. Immunol. 132:1501.

8. Mosser, D. M., J. F. Wedgwood, and P. J. Edelson. 1985. Leishmania amastigotes: resistance to complement-mediated lysis is not due to a failure to fix C3. J. Immunol. 134:4128.

9. Devine, D. V., R. J. Falf, and A. E. Balber. 1986. Restriction of the alternative pathway of human complement by intact Trypanosoma brucei subsp. gambiense. Infect. Immun. 52:223.

10. Joiner, K. A., N. Grossman, M. Schmetz, and L. Leive. 1986. C3 binds preferentially to long-chain lipopolysaccharide during alternative pathway activation by Salmonella montevideo. J. Immunol. 136:710.

11. Sacks, D. L., and P. V. Perkins. 1984. Identification of an infective stage of Leishmania promastigotes. Science (Wash. DC). 223:1417.

12. Franke, E. D., P. B. McGreevy, S. P. Katz, and D. L. Sacks. 1985. Growth cycle dependent generation of complement resistant Leishmania promastigotes. J. Immunol. 134:2713.

13. Joiner, K. A., S. Hieny, L. V. Kirchhoff, and A. Sher. 1985. gp72, the 72 kilodalton glycoprotein, is the membrane acceptor site for C3 on Trypanosoma cruzi epimastigotes. J. Exp. Med. 161:1196.