COMPLEMENT RECEPTOR TYPE 3 (CR3) BINDS TO AN Arg-Gly-Asp-CONTAINING REGION OF THE MAJOR SURFACE GLYCOPROTEIN, gp63, OF *LEISHMANIA* PROMASTIGOTES

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Leishmania is an obligate intracellular parasite that survives within the phagolysosomes of its vertebrate hosts' macrophages (1–3). Once the promastigote form of the parasite is introduced into the host tissue by a feeding sandfly, it must gain entry into a macrophage in order to survive and develop into the amastigote form. The initial attachment of *Leishmania* promastigotes to the macrophage is receptor mediated (4–6), however, the identity of the ligands on the parasite and their complementary receptors on the surface of the phagocyte is not known. Since some receptors on the macrophage surface initiate a microbicidal response when complexed with their ligand, and others do not (7–9), one possible means by which the parasite may increase its chance of successfully infecting the macrophage is in the "choice" of receptor(s) used during phagocytosis.

To date, two ligands on the surface of the promastigote capable of independently mediating attachment to the macrophage surface have been identified and isolated. These are the promastigote surface glycoprotein gp63 (10) and the promastigote lipophosphoglycan (11). The receptors for these specific ligands have not as yet been identified, but several studies conducted on intact parasites have implicated a variety of receptors that may function in the binding of promastigotes to macrophages. These receptors include the mannosyl/fucosyl receptor (5), a receptor for nonenzymatically glucosylated moeities (12), and the complement receptor type 3 (CR3) (13–15). Paradoxically, CR3 recognizes *Leishmania* promastigotes in the absence of exogenous complement. This led to the proposal that the macrophages itself secretes complement components that then deposit on the promastigote surface (13, 14). In the present study, we demonstrate that CR3 is the receptor for the promastigote surface glycoprotein gp63 and that CR3 binds directly to a region of gp63 containing the amino acid sequence Arg-Gly-Asp (R G D).

D. G. Russell is supported by a program grant from the MacArthur Foundation, and by grants from the Rockefeller Foundation, and National Institutes of Health Biomedical Research Support Grant SO7, RR-5399–26. S. D. Wright is supported by U. S. Public Health Service grants AI-22003 and AI-24775. S. D. Wright is an Established Investigator of the American Heart Association. Address correspondence to D. G. Russell, Dept. of Pathology, N. Y. U. Medical Center, 550 First Avenue, New York, NY 10016.

[1] *Abbreviations used in this paper:* CR3, complement receptor type 3; DPPC, dipalmitoylphosphatidylcholine; E, sheep erythrocytes; EA, E coated with antibody; EC3bi, E coated with C3bi complement components; MO, macrophages; PBS-NOG, PBS containing 10 mg/ml *n*-octyl glucoside.

Materials and Methods

Cells. All *Leishmania* lines were maintained as promastigotes in Semi-defined medium '79 (16) supplemented with 10% heat-inactivated FCS. The *Leishmania* strains used in this study were *L. mexicana* (MNYC/BZ/62/M379), *L. major* (NIH173,WR401), and *L. donovani* (DD8). All isolates were used for experiments within 8 wk of transformation from amastigotes, or from frozen stabilates of freshly transformed parasites.

Human monocytes were isolated from buffy coats on a Percoll gradient and cultured in RPMI plus 12.5% human serum in Teflon beakers as detailed previously (17). These cells were used after 4–10 d in culture and are referred to as macrophages (MO) in this study. Polymorphonuclear leukocytes were isolated from fresh human blood by the method of English and Anderson (18).

mAbs. The mAbs used in this study are described in Table I. TueL3.2 and TueL3.9 were present in ascites fluid, but all other antibodies were purified Igs. All the mAbs used were IgGs.

Isolation of gp63 and LPG. The promastigote glycoprotein gp63 was isolated by Con A chromatography of a detergent extract from a crude membrane preparation as described previously (10, 24). The gp63-containing fraction was then further fractionated by FPLC anion exchange chromatography on Mono Q-Sepharose, rather than the QAE Sephadex used in the earlier studies. The purity of all antigens was confirmed by SDS-PAGE before use. Soluble gp63, lacking the phospholipid anchor, was produced by incubating gp63 bound to Con A-Sepharose, with 5 U/ml phospholipase C from *Bacillus ceres* (type III; Sigma Chemical Co., St. Louis, MO). This phospholipase preparation is contaminated with PIPLC-specific lipase activity capable of removing gp63's PI anchor. After digestion, the enzyme and the cleaved tail were washed from the column, and the gp63 fraction was eluted as described above. Gp63 was further purified by anion exchange chromatography as described, in the absence of detergent.

The lipophosphoglycan was purified by differential solvent extraction by the procedure of Orlandi and Turco et al. (25, 26). Both gp63 and the lipophosphoglycan of *Leishmania* promastigotes (LPG) were isolated from stationary phase cultures.

Preparation of the Ligand-bearing Particles. Solid 5-μm diameter beads bearing defined ligands were made by a modification of the technique of Goldstein and Mescher (27), who used HPLC reverse-phase gel matrix as support for artificial membranes bearing class 1 antigens. In our hands, the lipid mixture used in the original paper rendered the particles "sticky" to MO. However, the substitution of the synthetic phospholipid dipalmitoylphosphatidylcholine (DPPC) for the original phospholipid mixture effectively reduced this behavior. DPPC has a phase transition temperature of 41°C, which means that at the experimental temperature, the lipid membrane is relatively immobile. This apparently reduces the likelihood of the bead surface binding or fusing with the MO membrane.

The coated particles, or "pseudocytes" (27), were prepared as follows. 25 mg ($\sim 5 \times 10^8$ particles) of 5 μm ODS1 reverse phase beads (Phase Separation) were suspended in 1 ml of PBS containing 10 mg/ml *n*-octyl glucoside (PBS-NOG). 10 mg of DPPC (Sigma Chemical Co.) was dissolved in 1 ml of PBS with 50 mg/ml *n*-octyl glucoside. The ligands, LPG and gp63, were then prepared at a 200-μg/ml concentration in PBS-NOG, and trace amounts of ligand metabolically labeled with [^{3}H]galactose (LPG) or [^{35}S]-methionine (gp63) were added to the preparations. In a typical preparation, 20 μl of the bead suspension was mixed with 25 μl of DPPC solution, ligand was then added to the desired concentration, and the total volume increased to 500 μl with extra PBS-NOG. The mixture was then placed in dialysis tubing from which all air was excluded. The tubing was anchored in a horizontal position to avoid unequal distribution of the beads and was then dialyzed at 4°C against PBS overnight. The beads were washed twice by gentle centrifugation and resuspended at 10^7/ml in fresh PBS. The percentage incorporation was calculated by counting the radioactivity in an aliquot of the beads. In preliminary experiments, the ratio of surface-exposed ligand vs. total incorporated ligand was calculated by incubating the beads in radiolabeled mAb and centrifuging the particles through a sucrose cushion. The percentage of exposed ligand was consistently ~60%.

Antibody-coated beads (BIgG) were prepared from the gp63-containing beads by incuba-

TABLE I
mAbs Used in This Study

mAb	Antigen	Reference
OKM10	α chain of CR3 (CD11b)	(23)
TS1/22	α chain of LFA-1 (CD11a)	(21)
LeuM5	α chain of p150,95 (CD11c)	(22)
IB4	β chain of CR3, LFA-1, p150,95 (CD18)	(23)
W6/32	HLA	(19)
A1A5	β chain of VLA antigens	(20)
TueL3.2	*Leishmania* gp63	(24)
TueL3.9	*Leishmania* gp63	(24)

tion of the particles in ascites fluid (2% in PBS) from two hybridoma cell lines, TueL3.2 and TueL3.9, secreting IgG1 antibodies against different epitopes on gp63.

Sheep erythrocytes (E) coated with antibody (EA) or C3bi (EC3bi) were prepared as described previously (17).

The Attachment of Ligand-bearing Particles to Human MO. Monolayers of MO were produced as follows. Cells were suspended at 10^6/ml in PBS with divalent cations (1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), 3 mM glucose, and 0.5 mg/ml human serum albumin. The cells were added in 5-μl aliquots to the wells of Terasaki tissue-typing plates and left to settle for 1 h at 37°C. In down-modulation experiments, the Terasaki wells had been previously coated with antibody by incubating the respective wells with 5 μl of PBS containing 25 μg/ml of the various anti-receptor mAbs for 1 h at 20°C. The monolayer was washed in PBS and rinsed in a final change of DGVB with divalent cations (2.5 mM veronal buffer, pH 7.5, 75 mM NaCl, 2.5% dextrose, 0.05 gelatin, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$). In experiments involving the use of soluble inhibitors, such as peptides, the inhibitors were introduced to the Terasaki plates at this point, immediately before the addition of the ligand-bearing particles.

Attachment of ligand-bearing particles to MO was determined by adding 5×10^4 coated beads or 2.5×10^5 erythrocytes per well and incubating for 15 or 45 min, respectively at 37°C. The attachment of the particles to the MO was then scored by phase contrast microscopy as detailed previously (17).

Synthetic Peptides. Peptide L10 (LGGALQAGDV), based on residues 402–411 of the γ chain of human fibrinogen, was synthesized by Peninsula. An inactive analogue of L10, AcL10, was prepared by acetylating lysine 406 as described (28). Both L10 and AcL10 were repurified by HPLC on a reverse-phase column. Peptide G15 (GQQHHLGGAKQAGDV) based on residues 398–411 of the γ chain of fibrinogen was purchased from Sigma Chemical Co. GRGDSP and GRGDESP were a generous gift from Dr. M. Pierschbacher, La Jolla, CA. Pep63 was synthesized from residues 365–385 of the gp63 amino acid sequence (RLLPGGLQQGRGDAVGPERGC) by Dr. D. Schlesinger, N.Y.U. Medical Center, NY.

Results

The Binding of gp63 Beads to Human MO. Purified gp63 was incorporated into a phospholipid monolayer supported by 5-μm beads. Fig. 1 illustrates the effect of increasing gp63 concentration on the binding of the beads to monolayers of MO. A density of 1.25×10^3 molecules/μm^2 was sufficient to facilitate the binding of at least one bead to 65% of the cells. Increasing the ligand density above this level caused a marked increase in the number of beads bound per MO. At a density of 6.35×10^3 molecules/μm^2, virtually all the MO had bound multiple beads (at least six beads per cell). All subsequent experiments to identify the MO receptor for gp63 were performed with beads containing $3–7 \times 10^3$ molecules/μm^2.

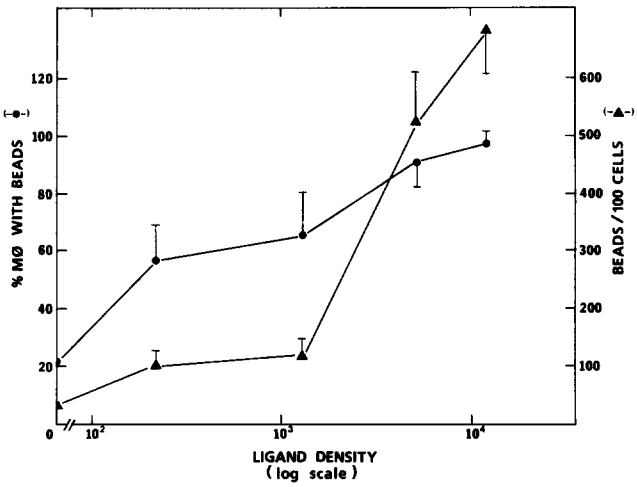

FIGURE 1. Binding of gp63 beads to MO. MO were incubated with beads bearing various densities of gp63, plotted above as molecules of gp63/ μm^2, and the percentage of cells with at least one attached bead and the total number of beads bound per 100 cells was measured. The vertical lines associated with each point indicate the SD. These values are the average from three independent experiments.

Phase-contrast microscopical examination of the MO after coincubation with the ligand-bearing beads revealed many gp63 beads bound to the surface of the phagocyte, but none appeared to have been internalized (Fig. 2 *A*). In contrast, gp63 beads incubated in IgG against gp63 before addition to the MO monolayers were phagocytosed by the MO (Fig. 2 *B*). Internalization of beads was also observed when the other promastigote ligand, LPG, was reconstituted onto the bead surface together with gp63 (data not shown). We therefore conclude that occupation of the receptor for gp63 is not sufficient stimulus to induce phagocytosis, at least at the ligand densities examined. The addition of another ligand, or a suitable opsonin such as IgG, to the bead surface triggers ingestion.

Down-modulation of Receptors on MO by mAb-coated Surfaces. MO were plated on tissue culture surfaces precoated with mAbs of known specificity. Previous work has shown that surface-bound mAbs cause depletion of target antigens from the accessible, upper portion of the cell plasmalemma (23). The attachment of gp63 beads was strongly inhibited by plating the MO onto antibodies specific for either the α chain (OKM10) or the β chain (IB4) of CR3 (Fig. 3). gp63-specific attachment, i.e., the binding attributable to the ligand on the bead surface, was inhibited by >85% by both these antibodies. In parallel studies, anti-CR3 antibodies mediated a similar inhibition of binding of EC3bi to the MO. Antibodies against the related proteins LFA-1 and p150,95 did not affect attachment of any particle. Similarly, antibodies against the β chain of the VLA family of antigens, which includes the fibronectin receptor, and against HLA molecules, did not affect the attachment of either gp63- or C3bi-bearing particles. None of the absorbed antibodies reduced the binding of antibody-coated gp63 beads, indicating that the reduction in attachment of gp63 beads by anti-CR3 antibodies was caused by depletion of accessible CR3.

The binding of ligand-bearing particles to MO plated onto human serum albumin, IgG, and collagen was also examined. None of these proteins altered the levels of binding of any of the particles. Fibronectin-coated surfaces, on the other hand, caused a small increase in the binding of both EC3bi and gp63 beads (data not shown) in a fashion consistent with previous observations on CR3-mediated attachment (29).

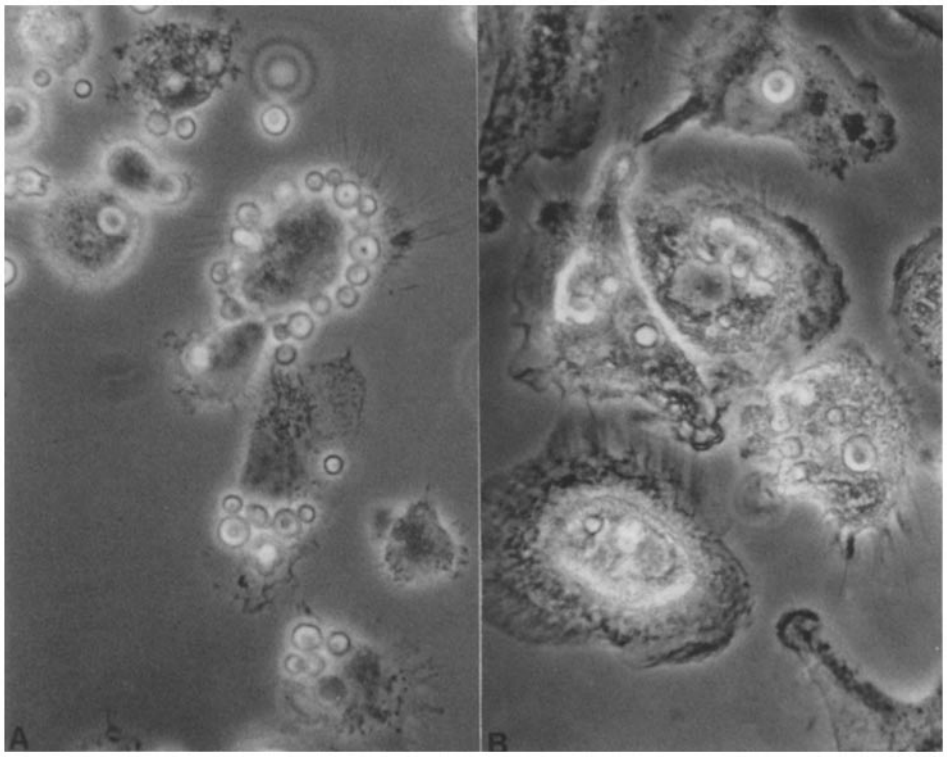

FIGURE 2. Binding and phagocytosis of ligand-bearing beads. Monolayers of MO were incubated with ligand-bearing beads for 15 min at 37°C as described in Materials and Methods. gp63 beads bind to the surface of cultured human MO, but no internalization is evident (*A*). However, gp63 beads coated with IgG anti-gp63 appeared to be phagocytosed by the MO (*B*).

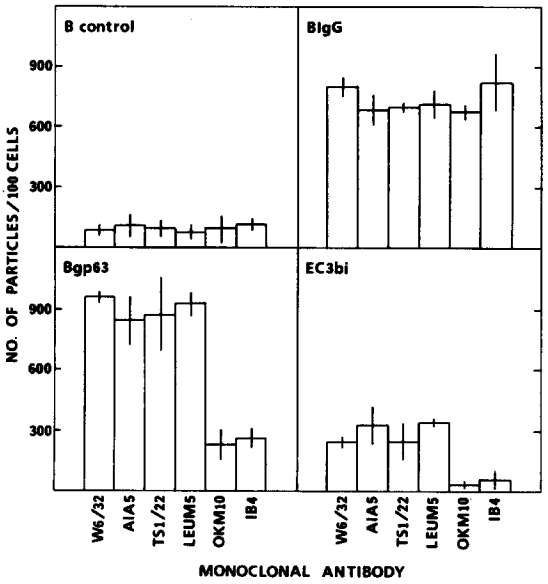

FIGURE 3. Modulation of receptors by surface-bound mAbs. Monolayers of MO were established on plastic surfaces coated with a variety of mAbs. These cells were then incubated together with ligand-bearing beads, and the number of beads bound per 100 cells were measured. The antibodies against HLA (*W6/32*), VLA (*A1A5*), LFA-1 (*TS1/22*), and p150/95 (*LEUM5*) had no effect on the attachment of any of the particles. Both antibodies against CR3 (*OKM10* and *IB4*) reduced the binding of EC3bi and gp63 beads (*Bgp63*). The attachment of beads without ligand (*Bcon*) and gp63 beads coated with IgG (*BIgG*) was unaffected by the antibodies. The vertical lines indicate the SD. These results were confirmed in six independent experiments.

TABLE II

The Sequence of Synthetic Peptides Used in This Study

Peptide designation	Sequence	Receptor inhibited
pep63	R L L P G G L Q Q G R G D A V G P E R G C	CR3
L10	L G G A K Q A G D V	CR3
AcL10	L G G A (acK) Q A G D V	No inhibition
G15	G Q Q H H L G G A K Q A G D V	CR3
GRGDSP	G R G D S P	FnR
GRGESP	G R G E S P	No inhibition

All these peptides were used in the competitive inhibition assay that is described in Figs. 4 and 5.

These results indicate that the fibronectin receptor is not involved in the binding of gp63 to the MO surface.

Gp63 beads also bound to polymorphonuclear leukocytes. As with the attachment to MO, this binding was mediated by CR3, and could be inhibited by soluble OKM10 antibody (data not shown).

Inhibition of gp63-mediated Binding to MO by Synthetic Peptides Containing Arg-Gly-Asp (R G D) and Related Sequences. CR3 is known to recognize a region of C3bi that contains the triplet Arg-Gly-Asp (R G D) (30). Since gp63 contains an R G D sequence at amino acid residues 375–377 (31), it is possible that CR3 could recognize this portion of the protein. We addressed this question experimentally by examining the effect of a synthetic 21-amino acid peptide, spanning residues 365–386 of the gp63 sequence (see Table II) on the binding of gp63 beads and EC3bi to MO.

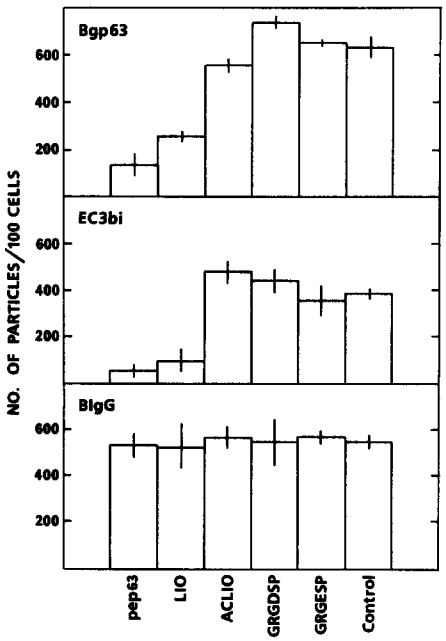

FIGURE 4. Competitive inhibition of binding of ligand-bearing beads with synthetic peptides. Monolayers of MO were established and incubated with ligand-bearing beads in the presence of different synthetic peptides. All peptides were added to a final concentration of 2 mg/ml. The peptides that inhibited binding of EC3bi also inhibited the attachment of gp63 beads (*Bgp63*). These peptides, pep63 and L10, are based on sequences from gp63 and fibrinogen, respectively (see Table II). None of the other peptides affected the attachment of any of the ligand-bearing beads. In addition, the gp63 beads coated with IgG (*BIgG*) and the beads without ligands (*Bcon*) were also unaffected by the peptides. These results were obtained in three separate experiments each run in duplicate.

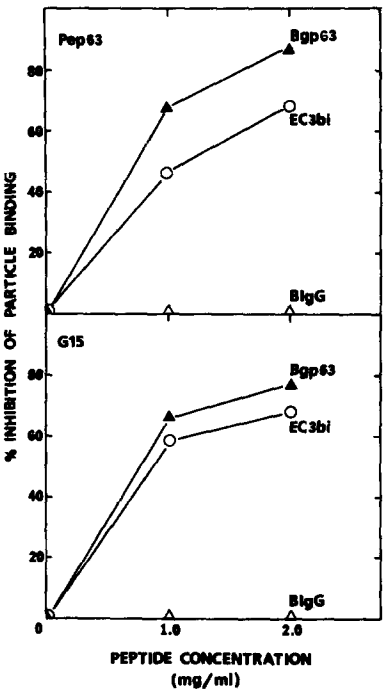

FIGURE 5. Inhibition of binding of ligand-bearing beads with synthetic peptides. Monolayers of MO were incubated with either EC3bi or gp63 beads (*Bgp63*) in the presence of increasing concentrations of synthetic peptides, pep63, and G15. Both peptides mediated comparable levels of inhibition of the binding of both particles. This graph shows the result from one experiment conducted in triplicate. Similar results were obtained in two independent experiments.

Soluble pep63 effectively inhibited the binding of both gp63 beads and EC3bi to MO (Fig. 4). Half-maximal inhibition was achieved at ~1 mg/ml (5×10^{-4} M) for both gp63 beads and EC3bi (Fig. 5). Parallel studies showed that, at the concentrations used, the peptide had no effect on the binding of IgG-coated gp63 beads, thus confirming the specificity of the observed inhibition. Moreover, synthetic peptides based on the R G D-containing region of fibronectin (32) failed to affect the binding of either gp63 beads or EC3bi. These data indicate that gp63 contains an amino acid sequence that interacts directly with CR3. In addition, these results confirm our finding that the fibronectin receptor does not function in the binding of gp63 by MO.

Recent studies have shown CR3 binds not only to the R G D-containing region of C3bi, but also to a related sequence in fibrinogen (Wright, et al., manuscript in preparation). CR3 recognizes the sequence K Q A G D V present in the fibrinogen γ chain, and peptides based on this sequence are particularly effective competitive inhibitors of EC3bi binding. The peptide L10 (L G G A K Q A G D V) inhibited the binding of both EC3bi and gp63 beads to MO (Fig. 4). In contrast, the acetylated form of the peptide AcL10, which had previously been shown to be unable to inhibit the binding of EC3bi (Wright, S.D., manuscript in preparation), had no effect on the ability of either gp63 beads or EC3bi to bind to MO (Fig. 4). We used a slightly longer peptide based on the fibrinogen sequence, G15, to demonstrate that the concentration of peptide required to produce effective inhibition of binding was comparable for both EC3bi and gp63 beads (Fig. 5). These findings further support the conclusion that the inhibition of binding of gp63 beads by synthetic peptides is due to competition for the complement binding site on CR3.

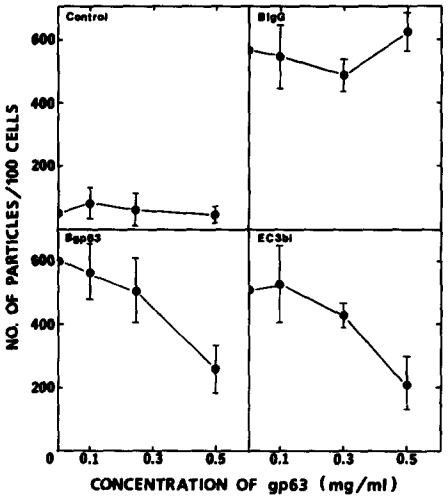

FIGURE 6. Inhibition of binding of ligand-bearing particles by soluble gp63. Monolayers of MO were incubated with ligand-bearing particles in the presence of increasing concentrations of gp63, made soluble by the removal of the phospholipid membrane anchor. gp63 inhibited the binding of both EC3bi and gp63 beads (*Bgp63*) to MO. Half-maximal inhibition of binding occurred at ~400 μg/ml of gp63 for both particles. gp63 had no effect on either the negative control, particles with no ligand added (*Control*), or the positive control, gp63 beads coated with IgG (*BIgG*). This graph shows the result from one experiment conducted in triplicate. Comparable results were obtained in three independent experiments.

The above data indicate that gp63 contains a sequence that can interact directly with CR3. To verify that the R G D-containing region is accessible on the native molecule, gp63 was made soluble by removal of the phospholipid anchor known to retain the protein in the membrane (33). Soluble gp63 competitively inhibited the attachment of both gp63 beads and of EC3bi to macrophages (Fig. 6). Half-maximal inhibition occurred at ~400 μg/ml (6.7×10^{-6} M). Thus on a molar basis, intact soluble gp63 is ~75-fold more effective than pep63 in inhibiting gp63 bead and EC3bi attachment.

The Effect of Soluble LPG and pep63 on the Binding and Uptake of Whole Promastigotes and Amastigotes. The attachment and ingestion of whole parasites by human MO in the presence of the promastigote surface antigen LPG and pep63 was examined. The parasites used included *L. mexicana* promastigotes, from both logarithmic and stationary phase cultures, and amastigotes. Both pep63 and LPG independently suppressed the attachment of promastigotes to the MO (Fig. 7). Simultaneous addition of both ligands caused enhanced inhibition, suggesting that the ligands occupy discrete binding sites. Similar results were obtained using *L. donovani* and *L. major* promastigotes, indicating that this dual recognition is not species specific (data not shown). Despite the marked effect these inhibitors had on the attachment of the promastigote stage, the binding of *L. mexicana* amastigotes was unaffected. This result demonstrates that the inhibition caused by pep63 and LPG in the promastigote experiments is unlikely to be due to toxicity, and that the amastigote possesses additional or alternative means of binding to and entering phagocytes.

Discussion

The participation of gp63 in the attachment of promastigotes to MO is known from previous studies, both in our own (10) and other laboratories (34). Since intact promastigotes possess an additional molecule (LPG) that also functions in attachment to MO (11), it has not been possible to dissect the individual contributions of these two components. We therefore developed a bead capable of carrying a single

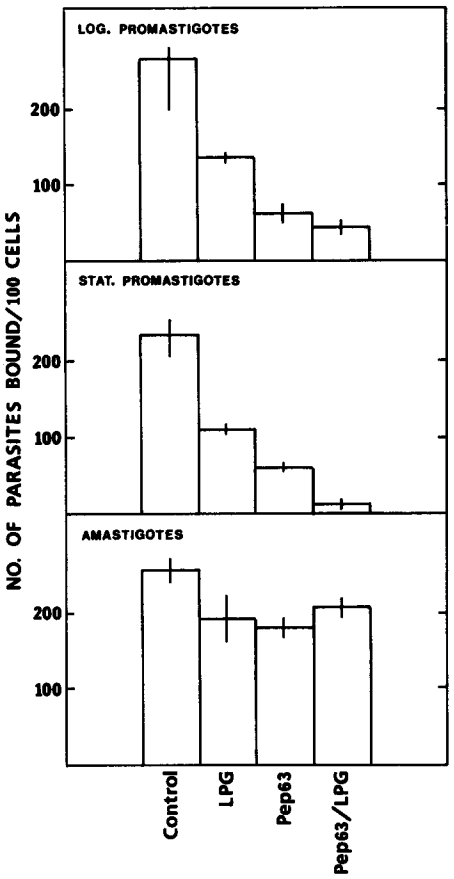

FIGURE 7. Inhibition of parasite attachment to MO by pep63 and LPG. Monolayers of MO were incubated with promastigotes from log or stationary phase cultures, or with amastigotes in the presence of LPG or pep63. The concentrations chosen, 2 mg/ml pep63 and 500 μg/ml LPG, were twice the concentration required to produce half-maximal inhibition in preliminary experiments. Both inhibitors depressed binding of promastigotes, an effect that was summated in preparations containing both ligands. Neither ligand had an effect on binding of amastigotes to MO. The values represent the average from two independent experiments run in triplicate.

promastigote ligand, gp63, and studied the interaction between the gp63-bearing particle and the MO surface. In the initial experiments, the binding of gp63 beads to MO was examined as a function of increasing ligand density (Fig. 1). Maximum binding occurred at ~6–7 × 10^3 molecules/μm^2, a density similar to the theoretical density of the ligand on the promastigote surface (6 × 10^3 molecules/μm^2). This was calculated assuming a cell surface area of 80 μm^2 and 500,000 copies/cell (35).

mAbs against CR3 have been reported to reduce the binding of *Leishmania* promastigotes to MO (13–15). Since CR3 expresses separate binding sites for both protein (C3bi) and for other ligands, such as bacterial LPS and *Histoplasma* (36, 37), the receptor could recognize *Leishmania* by binding gp63, LPG, or both. To determine if CR3 recognizes gp63, substrate-bound mAbs were used to selectively deplete CR3 from the upper surface of the cell. Such down-modulation of CR3 with either IB4 or OKM10 caused an 85% reduction in the binding of gp63 beads. A comparable inhibition of binding of EC3bi was observed in parallel experiments (Fig. 3). These results demonstrate that CR3 is the primary receptor responsible for binding gp63.

CR3 is a member of a large family of structurally homologous, adhesion-promoting receptors termed "integrins" (38, 39). This family includes the fibronectin receptor,

gpIIb/III of platelets, CR3, and the closely related leukocyte proteins LFA-1 and p150,95. Nearly all integrins bind ligands that contain R G D, or a similar amino acid sequence. For example, CR3 recognizes a region of C3bi that contains the R G D amino acid triplet (30). Since the deduced amino acid sequence of gp63 was found to contain the triplet R G D at residues 375–377 (31), the existence of which was predicted from immunological data (40), we asked whether this sequence was the target recognized by CR3. A synthetic peptide comprised of 21 amino acids spanning the R G D region of gp63 effectively inhibited the binding of both gp63 beads and EC3bi to MO (Figs. 4 and 5). Additional experiments demonstrated that peptides previously shown to inhibit the binding of EC3bi to MO were also able to inhibit the attachment of gp63 beads (Table II and Fig. 4). These results show that gp63 contains a sequence that binds directly to CR3, and that the binding site involved is the same binding site that recognizes C3bi.

To determine if the R G D-containing region of gp63 was accessible on the native molecule, we first removed the phospholipid membrane anchor. The resulting soluble gp63 competitively inhibited the attachment of both gp63 beads and EC3bi to MO (Fig. 6). Interestingly, gp63 was 75-fold more effective than pep63 at mediating inhibition. We assume that gp63 is a more avid ligand for CR3, because the intact protein holds the R G D-containing region in a favorable configuration.

Although gp63 is recognized by CR3, it is not bound by other integrins on the MO surface. Down-modulation of LFA-1, p150,95, or the fibronectin receptor by mAbs did not affect binding of gp63 beads (Fig. 3). Plating of the cells on fibronectin also failed to decrease binding. Finally, peptides based on the R G D-containing sequence of fibronectin (G R G D S P) did not affect the binding of gp63 beads (Fig. 4). The data is consonant with previous findings that CR3 does not recognize fibronectin or synthetic peptides based on the fibronectin sequence (30, 41). The specificity shown by gp63 for CR3 may, in consequence, aid in targetting the promastigote to the MO.

Other investigators have demonstrated that CR3 plays a role in the binding of whole promastigotes to macrophages (13–15). To explain the ability of antibodies against CR3 to block parasite binding in the absence of exogenous complement, Blackwell and coworkers (14) have proposed that MO secrete complement components that opsonize the promastigotes. While we cannot rule out a role for autoopsonization under some circumstances, several observations indicate that opsonization does not underlie the binding of gp63 to CR3 described in this study. Strong binding was observed within 15 min, during which time little secretion is likely to occur (42). Furthermore, we observed strong, anti-CR3-inhibitable binding to PMN, a cell type that has a very low rate of protein synthesis and has not been shown to produce C3. Finally, the binding of gp63 beads and EC3bi was inhibited by soluble gp63 (Fig. 6), presumably through competition for the same binding site on CR3. We therefore believe that a direct interaction between CR3 and gp63 is sufficient to explain the known CR3 dependence of binding of *Leishmania* promastigotes to MO.

Blackwell and colleagues (14) have also observed that Fab fragments from polyclonal anti-C3 antibody blocked the binding of promastigotes to MO and bound to the parasite surface during internalization (14). Since gp63 and C3bi contain a related amino acid sequence that is recognized by CR3, it is possible that the anti-C3 antibody bound to the R G D region of gp63, not C3. The existence of a cross-

reactive epitope is supported by current results showing that anti-pep63 antibody recognizes both gp63 and the α chain of C3 (Russell et al., manuscript in preparation).

Although our experiments demonstrate that CR3 binds gp63, it should be noted that gp63 beads bound by MO were not phagocytosed (Fig. 2). This finding is consistent with previous observations showing that CR3 on resting MO binds but does not cause internalization of EC3bi (17). These results indicate that promastigotes require additional, or alternative, ligands that bind to receptors on the MO surface to promote phagocytosis or to enable CR3 to initiate phagocytosis. A previous study has implicated LPG in the attachment of promastigotes to MO (11). Two observations in the present study support a role for LPG in host cell entry. The addition of LPG to the surface of gp63 beads induced the phagocytes to internalize the particles. Second, binding of promastigotes can be partially inhibited by soluble LPG, and this effect is additive with the inhibitory effect of pep63 (Fig. 7). The significance of gp63 and LPG as the principal ligands for MO is highlighted by their extreme abundance on the promastigote surface; 5×10^5 copies of gp63 (35) and 10^6 copies of LPG (Russell, D. G., unpublished results).

Leishmania promastigotes have been shown to differentiate in culture from a noninfective form of promastigote in actively dividing cultures, to an infective form in stationary phase cultures (43, 44). This differentiation is accompanied by changes on the promastigote surface. The surface expression of gp63 is increased (45), and LPG undergoes some structural modification (46) during the differentiation. Despite these changes, our inhibition experiments conducted with whole promastigotes (Fig. 7) indicate that both infective and noninfective stages use LPG and gp63 to attach to MO. This is consistent with previous observations showing that infectivity was not dependent on the ability of the parasite to bind to, and be internalized by, MO (44).

These results call into question the exact role of complement in the infection of MO by promastigotes. The predominant form of C3 deposited on the promastigote by serum is C3b, not C3bi (47), thus, raising the possibility that the C3b receptor (CR1) may also participate in the uptake of promastigotes opsonized with complement. Mosser and Edelson (48) have proposed that complement is necessary for the intracellular survival of *L. major* promastigotes. Our data, however, show that complement is not necessary for the binding of promastigotes to MO, and that gp63 binds directly to CR3. The relative contribution of complement, and the endogenous ligands gp63 and LPG, to the infection of MO is currently under study.

Summary

The major surface glycoprotein of *Leishmania* promastigotes, gp63, was isolated and reconstituted into a lipid membrane immobilized on the surface of 5-μm-diameter silica beads. These beads bound to the macrophage (MO), and the extent of binding correlated with the density of gp63 on the bead. The bead thus facilitated analysis of the binding specificity of a single ligand, gp63, without contribution from other molecules present on the surface of intact promastigotes. Plating of MO onto substrates coated with antibodies directed against several cell surface receptors indicated that the complement receptor CR3 was necessary for binding gp63.

CR3 recognizes a portion of C3 that contains the sequence R G D. Since gp63

also contains such a sequence, we tested the ability of a synthetic peptide based on the R G D-containing region of gp63 to inhibit the binding of gp63 beads. The R G D-containing peptide from gp63 inhibited the binding of both gp63 beads and EC3bi to MO. Similarly, peptides previously shown to inhibit the binding of C3bi also inhibited the attachment of gp63 beads. The synthetic peptide from the R G D region of gp63 also reduced the binding of intact promastigotes to MO. These results indicate that gp63 binds directly to CR3.

The authors acknowledge the expert technical assistance of Janis Zelechowski and Susan Levin and wish to thank Drs. Ralph M. Steinman and Pat A. Detmers for critical reading of the manuscript. D. G. Russell is grateful to Dr. V. Nussenzweig for his considerable contribution to the development and success of this study. The authors would also like to thank Dr. R. McMaster for communicating the unpublished sequence of gp63 to us and Dr. M. Hemler for his generous gift of A1A5 antibody.

Received for publication 14 March 1988.

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