

COMPLEMENT BINDING BY TWO DEVELOPMENTAL  
STAGES OF *LEISHMANIA MAJOR* PROMASTIGOTES  
VARYING IN EXPRESSION OF A SURFACE  
LIPOPHOSPHOGLYCAN

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The life cycle of *Leishmania* includes sequential development of invertebrate-stage promastigotes from a noninfective to an infective stage (1–4). This can be demonstrated for promastigotes growing both within the phlebotomine midgut and within axenic cultures. Promastigotes grown axenically to log stage are minimally infective for mice and are incapable of establishing intracellular infection within macrophages. In contrast, parasites grown to the stationary phase of growth demonstrate increased infectivity for cells and enhanced virulence for mice. The developmental changes occurring during transition from the noninfective log form to the infective metacyclic stationary phase form of *L. major* are current areas of study. Sacks et al. (5) have demonstrated that concentrations of peanut agglutinin (PNA)<sup>1</sup> that agglutinate 100% of organisms from log-phase cultures fail to agglutinate metacyclic promastigotes found within stationary cultures, and that the loss of agglutination with PNA can be used as the basis for purification of infective promastigotes (PNA<sup>-</sup>) from culture. This difference in PNA agglutinability reflects developmental modification of a prominent surface lipophosphoglycan (LPG) (6) previously shown by Handman et al. (7) to be shed from the parasite. Handman and Goding (8) have also demonstrated that the LPG, also termed excreted factor, is necessary for the attachment of the promastigote to macrophages (8), and that immunization of mice with this antigen confers protection to subsequent challenge (9). Studies by Sacks and da Silva (10) have shown that the developmentally regulated modification of the LPG involves acquisition of a novel carbohydrate determinant and that this modification appears to be a definitive marker for metacyclogenesis and a prerequisite for parasite infectivity in mice. Nonetheless, the mechanism by which this developmental change renders the parasite infective has not been defined.

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<sup>1</sup>Abbreviations used in this paper: C8D, C8-deficient serum; LPG, lipophosphoglycan; LOG, noninfective log-phase growth promastigotes; LS, lysis solution; NPGb, *p*-nitrophenyl-*o*-guanidino benzoate; PNA, peanut agglutinin.

Recent studies have focused attention on the interaction of the complement system with *Leishmania*. Franke et al. (11) have demonstrated that stationary phase promastigotes of various *Leishmania* species are relatively serum resistant, in comparison to the exquisite serum sensitivity demonstrated for promastigotes in the log phase of growth. Thus, *Leishmania* are similar to other trypanosomatids (12, 13), in that the infective form of the parasite within the vector is preadapted to a complement-resistant form before it comes in contact with the hostile environment of the vertebrate host complement system. However, the mechanism of this preadaptation in *Leishmania major* is poorly understood. There is recent evidence to suggest that not only are infective promastigotes resistant to killing by complement, but their interaction with the complement system may in fact promote their intracellular survival. Monocyte and macrophage receptors for the third component of complement are critical for *Leishmania* infectivity. Blackwell et al. (14) demonstrated that mAbs to the complement receptor CR3, which recognizes the iC3b fragment of complement, inhibited attachment of unfractionated *Leishmania donovani* promastigotes by nearly 80%. This group has suggested that complement components produced locally by macrophages are sufficient to opsonize the promastigote for complement receptor-mediated uptake (14), and have shown that stationary promastigotes of *L. donovani* bear more C3 on their surface (15). Mosser and Edelson (16) have extended the finding of Blackwell et al. (14) to *L. major*, and have shown that attachment and uptake are inhibited by 63% with anti-CR3 mAb and that intracellular survival can be enhanced by C3 opsonization (17).

Given the critical role that the complement system appears to play in the ability of *Leishmania* promastigotes to initiate infection, it was of obvious interest to compare complement binding by infective and noninfective developmental stages of these parasites. We have therefore undertaken a detailed analysis of the form of C3 on the surface of noninfective log-phase growth promastigotes (LOG) and metacyclic promastigotes of (PNA<sup>-</sup>) *L. major*, on the C3 acceptor molecules on these two stages, and on the processing of bound C3. Our results indicate that LOG and PNA<sup>-</sup> promastigotes activate complement through different pathways, but both forms bear predominantly C3b, which is associated with the LPG. Extensive release of C3b occurs from LOG but not PNA<sup>-</sup> promastigotes due to a proteolytic cleavage event. These results correlate with ongoing studies in our laboratory examining the complement receptors and ligands involved in cell attachment and invasion by *L. major*.

### Materials and Methods

**Parasites.** *L. major* Friedlin strain NIH, clone VI, (World Health Organization designation MHOM/IL/80/Friedlin) promastigotes were grown at 26°C in Grace's insect medium (Gibco Laboratories, Grand Island, NY) containing 20% (vol/vol) heat-inactivated FCS, 12 mM Hepes, 20 mM L-glutamine, 1,000 U/ml penicillin, and 50 µg/ml streptomycin. LOG were harvested when growth was at  $0.5-1 \times 10^7$  parasites/ml. Promastigotes not agglutinated by peanut agglutinin (PNA<sup>-</sup>) were harvested from a 5-6-d-old stationary phase culture ( $4-5 \times 10^7$  parasites/ml), as previously described (5). Briefly, PNA (Vector Laboratories, Inc., Burlingame, CA) was added to washed stationary growth promastigotes to a final concentration of 50 µg/ml. The PNA agglutinated parasites were separated from PNA nonagglutinated (PNA<sup>-</sup>) parasites by centrifugation at 150 g for 5 min at room temperature and were washed twice in HBSS<sup>2+</sup> (see below). LOG and PNA<sup>-</sup> promastigotes

were washed three times in HBSS<sup>2+</sup>, collected by centrifugation at 5,000 *g* for 20 min at room temperature, and maintained at room temperature until used in experiments.

**Buffers and Inhibitors.** HBSS (Gibco Laboratories) containing 1 mM MgCl<sub>2</sub> and 0.15 mM CaCl<sub>2</sub> (HBSS<sup>2+</sup>) was used for washing parasites and preparing serum dilutions. Protease inhibitors used throughout experiments were *p*-nitrophenyl-*o*-guanidino benzoate (NPGb) (Calbiochem-Behring Corp., La Jolla, CA) at a final concentration of 100 μM and leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, IN) at a final concentration of 25 μg/ml.

**Serum.** A pool of normal human serum (PNHS) was prepared from seven healthy donors. Serum deficient in complement component 8 (C8D) was obtained from a single donor. All serum aliquots were frozen at -70°C to maintain functional complement activity. Some samples of serum were heated to 56°C for 30 min to inactivate complement.

**Complement Components.** Human C3 was purified using minor modifications of the procedure previously described by Hammer et al. (18). Functionally active C3 was iodinated using Iodo-beads (Pierce Chemical Co., Rockford, IL) and <sup>125</sup>I-Na (Amersham Corp., Arlington Heights, IL) to a specific activity of 2.0–8.0 × 10<sup>5</sup> cpm/μg of total C3 protein. The functional hemolytic activity of the <sup>125</sup>I-C3 was ~25,000–50,000 C3 hemolytic Units/ml (60–75 C3 hemolytic U/μg).

**Antibodies and Solid-phase Immunoabsorbants.** Polyvalent goat anti-human C3 was prepared and the IgG fraction isolated using a two-step octanoic acid/DEAE-cellulose procedure previously described (18). Goat IgG anti-human C3 was coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described by Hammer et al. (18). Anti-human C3 antibody coupled to Sepharose 4B (anti-C3) recognized determinants on C3, C3b, iC3b, C3c, and C3dg. A rabbit polyclonal antibody directed against *L. major* gp63 was kindly provided by Dr. Clement Bordier (Institut de Biochimie, Université de Lausanne, Switzerland). An mAb against the LPG from LOG culture supernatants (79-3) was a gift from David Snary, Wellcome Biotechnology, Beckenham, United Kingdom. A purified monoclonal IgG1 fraction of 79-3 was prepared and covalently coupled to CNBr-Sepharose 4B-CL (Pharmacia Fine Chemicals) as suggested by the manufacturer. Antibodies linked to Sepharose were washed in wash solution (WS) (0.1% (vol/vol) Triton X-100 (Boehringer Mannheim Biochemicals), 38 mM Tris (Sigma Chemical Co., St. Louis, MO), 100 mM glycine (Schwarz/Mann, Cleveland, OH), pH 7.0), containing NPGb and leupeptin.

**Serum Killing.** LOG and PNA<sup>-</sup> promastigotes were incubated at 37°C for 60 min in PNHS at final serum concentrations ranging from 25% down to 0.18%. Percent killing was calculated relative to control samples incubated in heat-inactivated serum (see above). Killing was assessed by loss of promastigote motility and morphologic changes.

**C3 Binding Assay.** Binding of C3 to LOG and PNA<sup>-</sup> promastigotes during incubation in PNHS and C8D was determined. Promastigotes, at a final concentration of 5 × 10<sup>7</sup> parasites/ml in HBSS<sup>2+</sup>, were incubated at 37°C in 2.5, 5, 10, and 20% PNHS or 2.5 and 10% C8D to which was added <sup>125</sup>I-C3. Duplicate 200-μl aliquots of the mixture were removed at specific time points and layered over 1 ml of ice-cold HBSS<sup>2+</sup> in a 1.5-ml microcentrifuge tube and centrifuged in the cold at 12,000 *g* for 10 min. The supernatant was aspirated and <sup>125</sup>I-C3 within the pellet of parasites was determined. Total molecules of C3 bound/parasite was calculated at each time point based on the ratio of <sup>125</sup>I-C3 to unlabeled C3 present in each serum dilution, as previously described (19).

**C3 Uptake in Mg-EGTA Chelated C8D.** Binding of C3 to LOG and PNA<sup>-</sup> promastigotes via only the alternative pathway was determined. C8D serum was initially diluted in ice-cold HBSS<sup>2+</sup> containing 2 mM MgCl<sub>2</sub> and 10 mM EGTA and allowed to sit at 0° to 4°C for 15 min. LOG and PNA<sup>-</sup> promastigotes at a final concentration of 5 × 10<sup>7</sup> parasites/ml were incubated at 37°C in 10% C8D or 10% Mg-EGTA C8D to which was added <sup>125</sup>I-C3. Duplicate aliquots were removed at specific time points and were processed as described above to determine total molecules of C3 bound/parasite.

**C3 Fragmentation Pattern.** The form of C3 on the parasite surface during serum incubation was determined. A total of 2 × 10<sup>8</sup> LOG promastigotes or PNA<sup>-</sup> promastigotes were incubated for 30 min at 37°C in 20% C8D in HBSS<sup>2+</sup> to which was added <sup>125</sup>I-C3.

The final concentration of parasites was  $5 \times 10^7$  parasites/ml of reaction mixture. Parasites were collected by centrifugation at 12,000 *g* for 5 min in the cold, washed three times in HBSS<sup>2+</sup> containing NPGB and leupeptin, and the pellet of parasites was solubilized in 4% SDS, containing both inhibitors, by boiling at 100°C for 10 min. One-half of the volume of the SDS-solubilized serum-incubated parasites were brought to 2% SDS, 25 mM CH<sub>3</sub>NH<sub>2</sub>, 100 mM carbonate, pH 11.0, by the addition of a concentrated stock solution of methylamine and incubated at 37°C for 60 min. Methylamine treatment was done to cleave covalent ester linkages between C3 and parasite membrane constituents (20). The pH was neutralized to approximately pH 7 by the addition of 2–3  $\mu$ l of 5 N HCl, then samples were solubilized by boiling at 100°C for 10 min in SDS-sample buffer that contained 2-ME, NPGB and leupeptin. The remaining SDS-solubilized sample not treated with CH<sub>3</sub>NH<sub>2</sub> was boiled in SDS-sample buffer containing 2-ME and both inhibitors at 100°C for 10 min. SDS-PAGE was performed using 10% acrylamide slab gels by the method of Laemmli (21). Autoradiography was done using Cronex x-ray film (DuPont Co., Wilmington, DE) with a Quanta III enhancing screen (DuPont Co.).

*Analysis of Released C3 Fragments.* The C3 fragments released from the parasite surface after serum incubation were analyzed by SDS-PAGE autoradiography.  $5 \times 10^7$  LOG and  $5 \times 10^7$  PNA<sup>-</sup> parasites in HBSS<sup>2+</sup> were incubated in 1 ml of 10% C8D serum containing <sup>125</sup>I-C3 for 30 min at 37°C, washed twice in HBSS<sup>2+</sup> without inhibitors, and resuspended to the original volume. The mixture was incubated at 37°C. Aliquots were removed at various time points, centrifuged at 12,000 *g* for 5 min at 4°C and the supernatant was removed. Both supernatant and pellet of parasites were solubilized in SDS-sample buffer containing NPGB, leupeptin, and 2% 2-ME by boiling at 100°C for 10 min. SDS-PAGE autoradiography was done as described above.

*C3c Release Assay.* The percentage of the C3b and iC3b on the parasite surface during serum incubation was also determined by an alternative approach. We performed this analysis using a modification of the method previously described by Gaither et al. (22). This procedure provides a quantitative method of determining the distribution of membrane-bound C3b and iC3b, based on release by low concentrations of trypsin; iC3b is sensitive to trypsin proteolysis while C3b is relatively resistant to limiting concentrations of trypsin.  $2 \times 10^8$  LOG promastigotes or PNA<sup>-</sup> promastigotes were incubated for 30 min at 37°C in 20% C8D in HBSS<sup>2+</sup> to which was added <sup>125</sup>I-C3. The final concentration of parasites was  $5 \times 10^7$  parasites/ml of reaction mixture. Parasites were collected by centrifugation at 12,000 *g* for 5 min at 4°C, washed three times in ice-cold HBSS<sup>2+</sup> without inhibitors, and resuspended in 1 ml ice-cold HBSS<sup>2+</sup>. 100  $\mu$ l of washed serum-incubated parasites were combined with 100  $\mu$ l of a dilution of TPCK trypsin (Sigma Chemical Co., serial fivefold dilution from 2.5 mg/ml to 0.8  $\mu$ g/ml) prepared in HBSS<sup>2+</sup>, and incubated at 30°C for 10 min. 50  $\mu$ l of ice-cold inhibitor solution containing 16 mg soybean trypsin inhibitor (Sigma Chemical Co.) per milliliter HBSS<sup>2+</sup>, 50  $\mu$ M NPGB, and 2.5  $\mu$ g/ml leupeptin was added to inhibit trypsin proteolysis. All samples were then centrifuged at 12,000 *g* for 10 min in the cold and the supernatant was immediately removed. Both pellet and supernatant were solubilized in SDS sample buffer as described above. <sup>125</sup>I-C3 counts in supernatant and pellet were determined and the percent <sup>125</sup>I-C3 counts released into the supernatant was calculated. SDS-PAGE autoradiography of the supernatants was performed as described above.

*Determination of C3 Acceptor by Immunoprecipitation.* The molecule to which C3 binds on LOG and PNA<sup>-</sup> parasites was determined. Native parasites were incubated in serum containing <sup>125</sup>I-C3, and the extent of precipitation of <sup>125</sup>I-C3 from detergent-solubilized parasites was compared for antibodies directed against the LPG and gp63. LOG and PNA<sup>-</sup> promastigotes were incubated in C8D containing <sup>125</sup>I-C3 for 30 min at 37°C at a final concentration of  $5 \times 10^7$  parasites/ml. The serum-incubated parasites were washed twice and the pellet of parasites ( $2 \times 10^8$ ) that was collected by centrifugation at 12,000 *g* for 5 min was solubilized overnight at 4°C in 1 ml of LS (1% vol/vol Triton X-100; Boehringer Mannheim Biochemicals), 38 mM Tris (Sigma Chemical Co.), 100 mM glycine (Schwarz/Mann), pH 7.0, containing NPGB and leupeptin. This lysate was cleared by centrifugation at 12,000 *g* for 10 min in the cold and preabsorbed for 4 h with 50  $\mu$ l

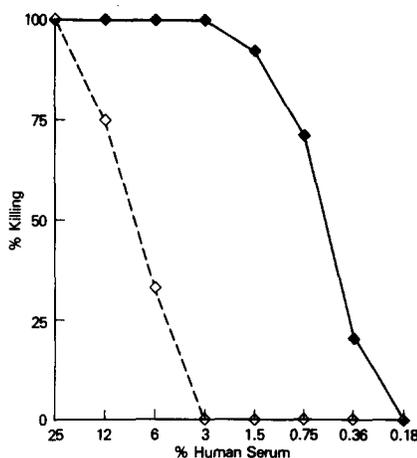


FIGURE 1. Killing of LOG and PNA<sup>-</sup> promastigotes in normal human serum. LOG (solid line) and PNA<sup>-</sup> (broken line) promastigotes at a final concentration of  $2 \times 10^7$ /ml were incubated for 60 min at 37°C in dilutions of PNHS ranging downward from 25 to 0.18%. Percent killing was determined by light microscopic assessment of shape and motility.

(packed volume) Sepharose 4B that had been swelled and washed in lysis solution (LS). The lysate was centrifuged at 12,000 *g* for 30 s and the supernatant was removed. 100  $\mu$ l (packed volume) of immunoabsorbant 79-3 suspended in WS (0.1% Triton X-100, 38 mM Tris, 100 mM glycine, pH 7.0) was added to a 200- $\mu$ l aliquot of cleared lysate that had been diluted 10-fold with WS containing NPGB and leupeptin, rocked overnight at 4°C. 25  $\mu$ l of anti-gp63 or prebleed serum from the same rabbit was added to similarly prepared samples of cleared LOG and PNA<sup>-</sup> lysates then rocked for 6 h at 4°C, followed by the addition of 100  $\mu$ l (packed volume) of protein A-Sepharose 4B (Pharmacia Fine Chemicals) and rocked overnight at 4°C. Total <sup>125</sup>I-C3 cpm bound by the immunoabsorbants was determined and the percent <sup>125</sup>I-C3 immunoprecipitated calculated relative to input counts.

Experiments were done using <sup>125</sup>I-labeled LOG and PNA<sup>-</sup> promastigotes to assess the efficiency of immunoprecipitation by anti-gp63.  $3 \times 10^8$  LOG and PNA<sup>-</sup> promastigotes were <sup>125</sup>I labeled as previously described (23) with 500  $\mu$ Ci <sup>125</sup>I-Na (Amersham Corp.) and incubated in 10% C8D at a final concentration of  $5 \times 10^7$  parasites/ml for 30 min at 37°C. Lysates were prepared and immunoprecipitated with anti-gp63 and prebleed serum as described.

The molecular form of C3 associated with the C3 acceptor that was immunoprecipitated by 79-3 and anti-C3 was examined by SDS-PAGE autoradiography. Lysates of LOG and PNA<sup>-</sup> promastigotes bearing <sup>125</sup>I-C3 after incubation in C8D serum were prepared and immunoprecipitated with 100  $\mu$ l (packed volume) of 79-3 or anti-C3 immunoabsorbant. <sup>125</sup>I-C3 counts were eluted by boiling at 100°C for 10 min in 110  $\mu$ l of 4% SDS-sample buffer containing 2-ME, NPGB, and leupeptin. SDS-PAGE autoradiography was done as described above.

## Results

**Serum Killing.** LOG and PNA<sup>-</sup> promastigotes were examined for their respective sensitivity or resistance to serum killing (Fig. 1). 100% of LOG promastigotes were killed when incubated in serum dilutions ranging from 3 to 25%. Below 3% serum concentration, only a fraction of the LOG promastigotes were killed after incubation for 60 min at 37°C. This is in contrast to PNA<sup>-</sup> promastigotes that were partially resistant to serum-mediated killing at serum concentrations from 25 to 3%, but completely resistant to serum killing at serum concentrations of 3% or less.

**C3 Uptake in PNHS.** Quantitative measurement of the binding of C3 to LOG

and PNA<sup>-</sup> promastigotes was initially done in kinetic fashion in varying dilutions of PNHS (Fig. 2). At 15 min of serum incubation there was substantial binding of C3 to LOG promastigotes at each serum dilution. Maximum binding of C3 at each serum dilution was greater for LOG promastigotes than for PNA<sup>-</sup> promastigotes. Additionally, there was a loss of total molecules of C3 bound to LOG at 30, 60, 90, and 120 min of serum incubation when compared with the 15-min time point. This spontaneous loss of C3 was substantially greater for LOG than for PNA<sup>-</sup> promastigotes. The kinetics of C3 binding to the LOG promastigote surface correlated with the kinetics of serum killing (not shown). For PNA<sup>-</sup>, no killing occurred at 60 min in 3% serum, despite binding of  $2.3 \times 10^5$  molecules C3/parasite in 2.5% PNHS. In 5% serum, only 20% killing of PNA<sup>-</sup> promastigotes was achieved at 60 min, despite binding of  $4.4 \times 10^5$  molecules C3/parasite, a level that led to 100% killing of LOG promastigotes. These results suggest that serum resistance in PNA<sup>-</sup> does not simply reflect inefficient complement activation.

*C3 Uptake in C8D.* A serum source deficient in terminal complement component 8 (C8D) was also used to examine the binding of C3 to the surface of the LOG or PNA<sup>-</sup> promastigotes. C3 bound rapidly to LOG and PNA<sup>-</sup> promastigotes with maximal C3 binding occurring at the 15-min time point for the LOG and at the 90-min point for PNA<sup>-</sup> when incubated in 2.5% C8D (Fig. 3). In the absence of an intact terminal complement cascade, no killing was observed for LOG or PNA<sup>-</sup> (data not shown), indicating that release of bound C3 was not a consequence of parasite lysis.

*C3 Uptake Via the Alternative Pathway.* Mg-EGTA C8D was used to determine quantitatively the binding of C3 on LOG and PNA<sup>-</sup> promastigotes that occurs via the alternative pathway. As shown in Fig. 4, LOG promastigotes bind C3 in Mg-EGTA C8D to an extent nearly equivalent to that in nonchelated serum. There is a lag in the onset of C3 deposition, consistent with activation through the alternative pathway. In contrast, PNA<sup>-</sup> promastigotes bind negligible amounts of C3 during 120 min of incubation in Mg-EGTA C8D, indicating that PNA<sup>-</sup> parasites do not activate the alternative pathway in nonimmune serum. Similar results were obtained when PNHS was used instead of C8D.

*Molecular Form of C3.* We analyzed serum-incubated promastigotes bearing <sup>125</sup>I-C3 for the molecular form of C3. The predominant C3  $\alpha'$  fragment-acceptor complex on both the LOG and PNA<sup>-</sup> promastigotes (Fig. 5, - lanes) migrates as a diffuse band of molecular weight 140–340  $\times 10^3$  (LOG) and 200–340  $\times 10^3$  (PNA<sup>-</sup>). Since the maximum size of the native  $\alpha'$  chain of C3b is 110  $\times 10^3$  mol wt, this result indicates that C3 binds covalently to a parasite membrane C3 acceptor. Of note, there is a prominent 110  $\times 10^3$  mol wt band only on the surface of the PNA<sup>-</sup> promastigote.

We next analyzed the molecular form of the bound C3 fragments of serum incubated LOG and PNA<sup>-</sup> promastigotes after treatment with methylamine, a nucleophile that cleaves *O*-ester linkages (Fig. 5, + lanes). The <sup>125</sup>I-C3 fragments generated from LOG and PNA<sup>-</sup> promastigotes by methylamine treatment migrated at 110 and 68  $\times 10^3$  mol wt, corresponding to C3b and iC3b, respectively. The major fragment of C3 after methylamine treatment on both parasites was 110  $\times 10^3$  mol wt. These experiments show that both LOG and PNA<sup>-</sup> promas-

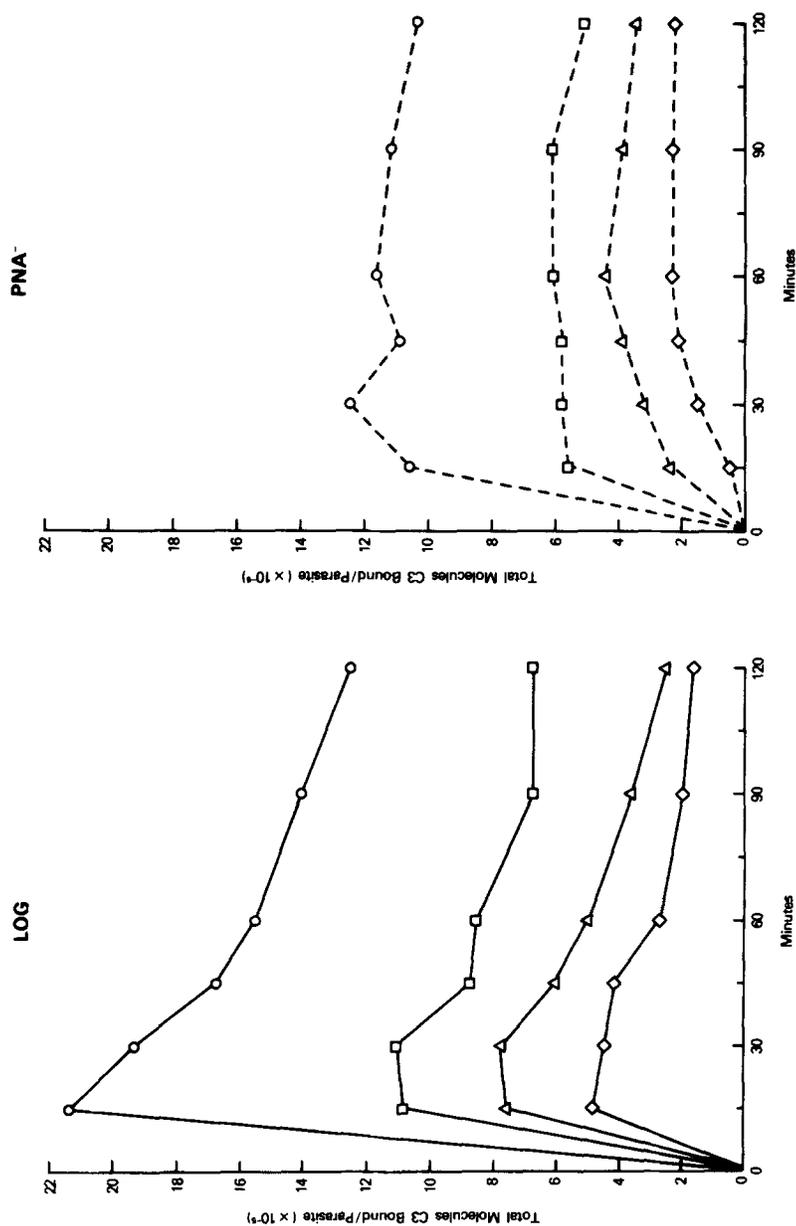


FIGURE 2. Kinetics of C3 binding to LOG or PNA<sup>-</sup> promastigotes in PNHS. 5 x 10<sup>7</sup> LOG (—) promastigotes/ml or 5 x 10<sup>7</sup> PNA<sup>-</sup> (---) promastigotes/ml were incubated at 37°C for 120 min in 2.5% (◇), 5% (Δ), 10% (□), and 20% (○) PNHS containing <sup>125</sup>I-C3. Total molecules C3 bound per parasite was determined at indicated time points.

FIGURE 2. Kinetics of C3 binding to LOG or PNA<sup>-</sup> promastigotes in PNHS. 5 x 10<sup>7</sup> LOG (—) promastigotes/ml or 5 x 10<sup>7</sup> PNA<sup>-</sup> (---) promastigotes/ml were incubated at 37°C for 120 min in 2.5% (◇), 5% (Δ), 10% (□), and 20% (○) PNHS containing <sup>125</sup>I-C3. Total molecules C3 bound per parasite was determined at indicated time points.

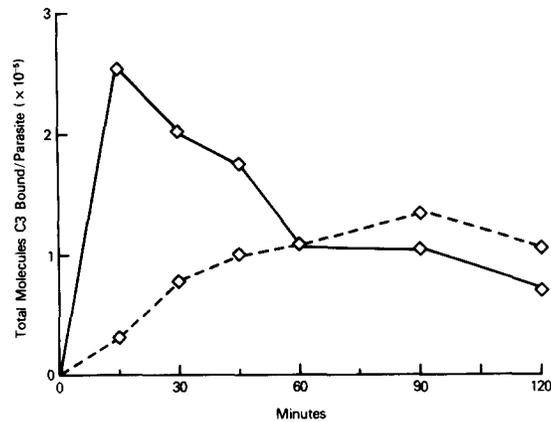


FIGURE 3. Kinetics of C3 binding to LOG and PNA<sup>-</sup> promastigotes in C8D serum.  $5 \times 10^7$  LOG (—) promastigotes/ml or  $5 \times 10^7$  PNA<sup>-</sup> (---) promastigotes/ml were incubated at 37°C for 120 min in 2.5% C8D containing <sup>125</sup>I-C3. Total molecules of C3 bound per parasite was determined at indicated time points.

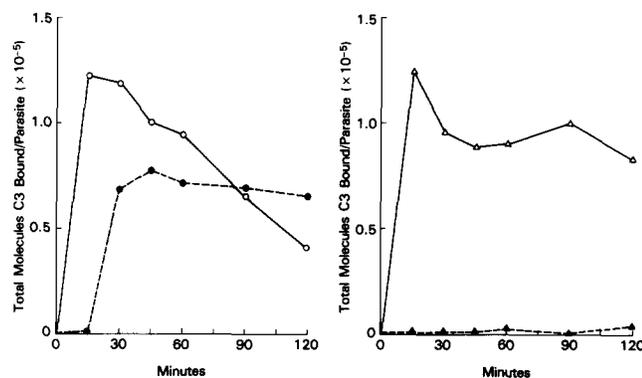


FIGURE 4. Kinetics of C3 binding to LOG and PNA<sup>-</sup> promastigotes in C8D serum and Mg-EGTA C8D serum.  $5 \times 10^7$  LOG (○, ●) promastigotes/ml and  $5 \times 10^7$  PNA<sup>-</sup> (△, ▲) promastigotes/ml were incubated at 37°C for 120 min in either 10% C8D (—) or 10% Mg-EGTA C8D (---) containing <sup>125</sup>I-C3. The 10% Mg-EGTA C8D incubation was to limit complement activation to the alternative pathway. Total molecules C3 bound per parasite was determined at indicated time points.

tigotes bear predominantly C3b, that before methylamine treatment PNA<sup>-</sup> promastigotes bear a large amount of C3 in a molecular form of  $110 \times 10^3$  mol wt, and that the majority of C3 that is covalently attached to parasite membrane C3 acceptors is bound by a cleavable *O*-ester linkage.

**C3c Release Results.** We wanted to confirm the distribution of C3 fragments on LOG and PNA<sup>-</sup> parasites using a different approach. Whereas iC3b is sensitive to proteolysis at low concentrations of trypsin, resulting in release of C3c into the supernatant, C3b is resistant to proteolysis (22). We found that 0.4 μg/ml of trypsin released 11.9% of <sup>125</sup>I-C3 from serum-incubated LOG and 15.0% of the <sup>125</sup>I-C3 from the serum-incubated PNA<sup>-</sup> promastigotes. The expected presence of the C3c fragment in the trypsin supernatant was confirmed by SDS-PAGE autoradiography (Fig. 6) in which a  $27 \times 10^3$  mol wt band corresponding to the labeled α' fragments of C3c was generated in limiting trypsin dilutions. Also of note, corroborating results in Fig. 5, is the  $110 \times 10^3$  mol wt band in the PNA<sup>-</sup> pellet but not the LOG pellet. These results confirmed the SDS-PAGE analysis of bound C3 fragments (Fig. 5), showing that >80% of the C3 on the surface of both stages of promastigotes is in the form of C3b.

**Spontaneous Release of C3.** We next examined the <sup>125</sup>I-C3 fragments released

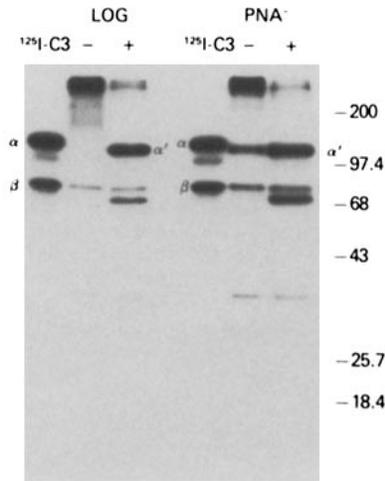


FIGURE 5. Molecular form of C3 on *L. major* promastigotes. LOG and PNA<sup>-</sup> promastigotes at  $5 \times 10^7$ /ml were incubated for 30 min at 37°C in 20% C8D containing <sup>125</sup>I-C3. Promastigotes were washed, then solubilized at 100°C in 4% SDS containing NPGB and leupeptin. A portion of the solubilized parasites were then incubated with 25 mM CH<sub>3</sub>NH<sub>2</sub> to cleave covalent O-ester linkages between C3 and parasite constituents (+ lanes) before solubilization in SDS-sample buffer. Non-methylamine-treated parasites (- lanes) were solubilized directly in SDS-sample buffer. Samples were analyzed by 10% SDS-PAGE autoradiography.

- No Methylamine Treatment  
+ Methylamine Treatment

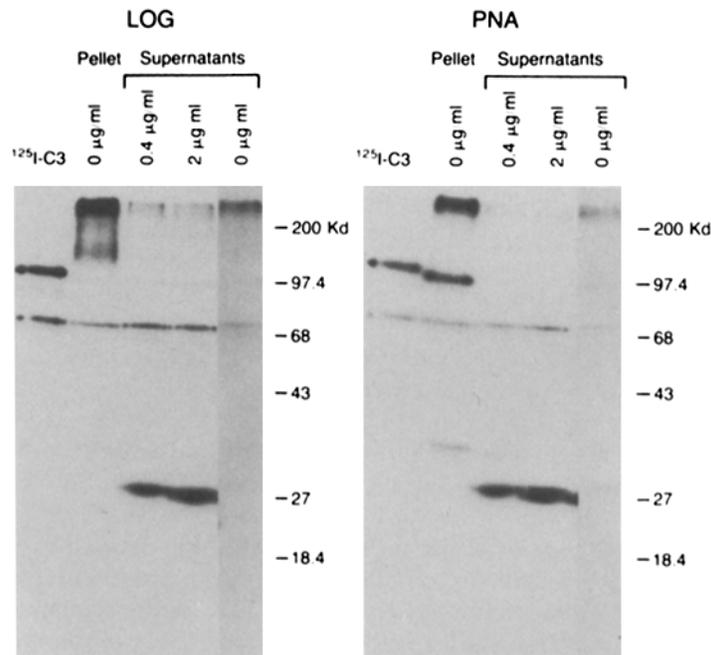


FIGURE 6. SDS-PAGE analysis of <sup>125</sup>I-C3 fragments released from serum-incubated LOG and PNA<sup>-</sup> promastigotes treated with limiting dilutions of trypsin.  $2 \times 10^7$  LOG and PNA<sup>-</sup> promastigotes were incubated in 20% C8D then washed. Aliquots containing  $5 \times 10^6$  promastigotes were incubated in dilutions of trypsin (0, 0.4, and 2 µg/ml final concentration). LOG and PNA<sup>-</sup> parasite pellets without trypsin treatment and the supernatants of the trypsin treated LOG and PNA<sup>-</sup> parasites were analyzed by 10% SDS-PAGE autoradiography under reducing conditions.

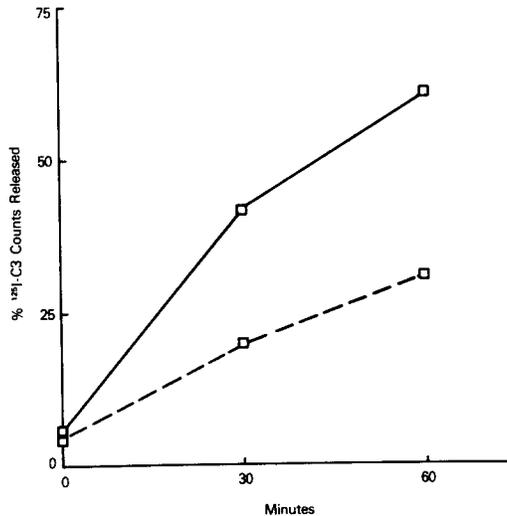


FIGURE 7. Spontaneous release of  $^{125}\text{I}$ -C3 by *L. major* promastigotes after incubation in C8D serum.  $5 \times 10^7$  LOG and  $5 \times 10^7$  PNA<sup>-</sup> promastigotes were incubated for 30 min in 10% C8D containing  $^{125}\text{I}$ -C3. The parasites were washed in HBSS<sup>2+</sup> and incubated in HBSS<sup>2+</sup> for 60 min at 37°C. Percent of total  $^{125}\text{I}$ -C3 counts released spontaneously into buffer by LOG (—) and PNA<sup>-</sup> (---) was determined at 0, 30, and 60 min.

from the parasite surface after serum incubation. Parasites bearing  $^{125}\text{I}$ -C3 were washed to remove serum then incubated in HBSS<sup>2+</sup> buffer. There was continued spontaneous release of  $^{125}\text{I}$ -C3 by both LOG and PNA<sup>-</sup> promastigotes after serum incubation with nearly a threefold greater percentage of release of  $^{125}\text{I}$ -C3 fragments by LOG promastigotes (Fig. 7). Analysis by SDS-PAGE of the  $^{125}\text{I}$ -C3 fragments released into serum-free buffer (Fig. 8, S lanes) revealed the generation of multiple fragments of  $^{125}\text{I}$ -C3 of molecular weight 68, 35, and  $25 \times 10^3$ . The  $68 \times 10^3$  mol wt fragment represents the  $\alpha'$ -chain of iC3b. The  $35 \times 10^3$  and  $25 \times 10^3$  mol wt  $^{125}\text{I}$ -C3 fragments are atypical C3 cleavage fragments when compared with the scheme for physiologic C3 breakdown by factor I and factor H, or CR1<sup>24</sup> (reviewed in reference 24). No high molecular weight C3-acceptor complexes are released into the supernatant and spontaneous release of intact C3b from the parasite C3 acceptor is negligible. These results show that release of C3 from both LOG and PNA<sup>-</sup> is a consequence of proteolytic cleavage.

**Determination of C3 Acceptor.** Experiments were performed to define the parasite molecule to which C3 was covalently attached, the C3 acceptor molecule. Parasites were incubated in serum containing  $^{125}\text{I}$ -C3, and the extent of immunoprecipitation of  $^{125}\text{I}$ -C3 fragments with antiparasite antibodies was determined. Direct immunoprecipitation of detergent solubilized LOG and PNA<sup>-</sup> promastigotes bearing  $^{125}\text{I}$ -C3 with mAb 79-3, directed against both LOG and PNA<sup>-</sup> LPG, showed in five separate experiments that the LPG defined by this mAb is the major C3 acceptor. Table I shows the data from a representative experiment in which 79-3 immunoprecipitated 87.4% and 67.7% of the covalently attached  $^{125}\text{I}$ -C3 to LOG and PNA<sup>-</sup>, respectively. In contrast, anti-gp63 immunoprecipitated <11% of labeled C3 on both LOG and PNA<sup>-</sup> promastigotes. In a control experiment, the majority of  $^{125}\text{I}$ -labeled gp63 was immunoprecipitated under similar conditions.

The molecular form of C3 fragments immunoprecipitated by 79-3 was examined by SDS-PAGE autoradiography. The pattern was compared with the fragments recognized by anti-C3 to determine if a particular subset of C3 fragments

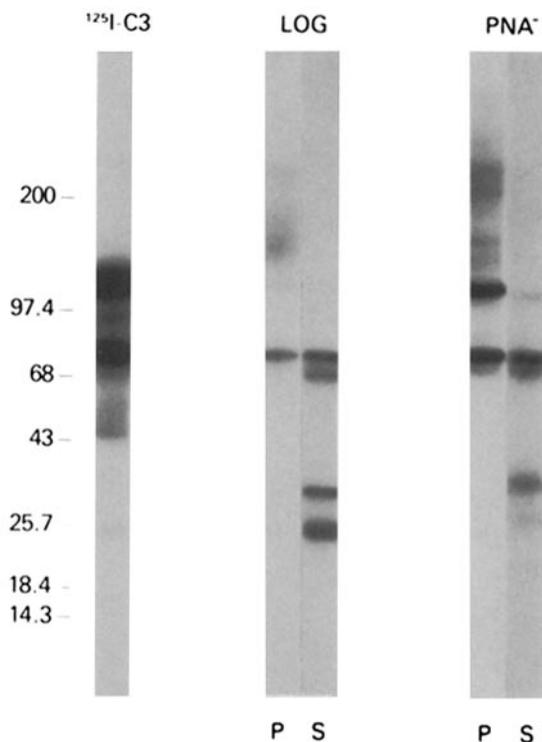


FIGURE 8. SDS-PAGE autoradiography of <sup>125</sup>I-C3 fragments spontaneously released from *L. major* promastigotes after 60 min of incubation in buffer.  $5 \times 10^7$  LOG and  $5 \times 10^7$  PNA<sup>-</sup> promastigotes were incubated for 30 min in 10% C8D containing <sup>125</sup>I-C3. The parasites were washed in HBSS<sup>2+</sup> and incubated in HBSS<sup>2+</sup> for 60 min at 37°C. The form of released C3 and of residual bound C3 was determined by 10% SDS-PAGE autoradiography. P, pellet; S, supernatant.

TABLE I  
*Determination of C3 Acceptor by Immunoprecipitation*

<i>L. major</i> stage	<sup>125</sup> I-C3 counts immunoprecipitated by:		
	79-3	anti-gp63	Prebleed
		%	
LOG	87.4	5.7	5.4
PNA <sup>-</sup>	67.7	10.8	8.7

was associated with the LPG. The molecular form of C3 associated with the LPG (Fig. 9 A) was similar to that on whole parasites (Fig. 5) and to the fragments immunoprecipitated by anti C3 (Fig. 9 B). In LOG, the major band immunoprecipitated with 79-3 was a high molecular weight C3-acceptor complex whereas a high molecular weight C3-acceptor complex was present along with a prominent  $\alpha'$   $110 \times 10^3$  mol wt band in PNA<sup>-</sup>. These results thus indicate that the fragmentation profile of C3 bound to the LPG acceptor reflects the profile of total C3 on the parasite surface (Fig. 5).

### Discussion

We have examined the activation of complement by two developmental stages of *L. major* promastigotes: noninfective, serum-sensitive LOG-phase promastigotes and infective, serum-resistant stationary-phase PNA<sup>-</sup> promastigotes. Quan-

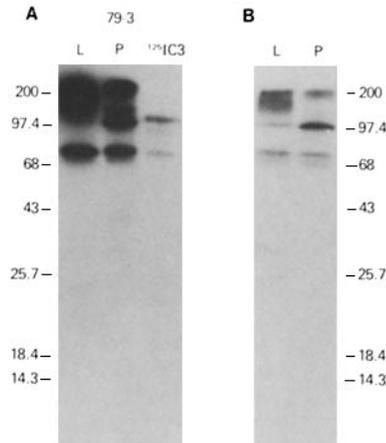


FIGURE 9. Determination of the C3 acceptor by SDS-PAGE analysis of C3 fragments associated with LPG. Samples of  $^{125}\text{I}$ -C3 fragments immunoprecipitated with anti-LPG mAb 79-3 (A) and anti-human C3 (B) were solubilized in SDS-sample buffer containing 2-Me and analyzed by 5–15% SDS-PAGE autoradiography. L, LOG; P, PNA<sup>-</sup>. Mol wt markers ( $\times 10^{-3}$ ) are shown.

titative measurement of the binding of C3 to LOG promastigotes in PNHS and C8D serum correlates with the kinetics of serum killing, whereas for PNA<sup>-</sup> there is no such correlation. LOG and PNA<sup>-</sup> promastigotes activate complement rapidly as determined by  $^{125}\text{I}$ -C3 binding studies, but a distinction between the two developmental stages is apparent when the complement pathway being activated and the molecular form of C3 on the surface are compared. Whereas LOG parasites activate the alternative pathway efficiently, PNA<sup>-</sup> forms are totally incapable of activating the alternative pathway in human serum. Both LOG and PNA<sup>-</sup> promastigotes bear primarily hemolytically active C3b on their surface; however a  $110 \times 10^3$  mol wt C3 fragment is found associated only with the infective PNA<sup>-</sup> promastigotes. Nonetheless, the major C3 acceptor for both LOG and PNA<sup>-</sup> is the LPG. Thus, serum resistance in PNA<sup>-</sup> promastigotes does not result from inefficient complement activation through C3 nor a failure to generate hemolytic C3 fragments on the parasite surface. Instead, changes in the parasite surface, possibly related to expression of the developmentally regulated LPG, must affect lytic complement activity at a later step in the cascade.

LOG parasites activated the alternative pathway efficiently in comparison to a complete absence of alternative pathway activation by PNA<sup>-</sup> promastigotes. No analogous observation has been made previously with any *Leishmania* species. Earlier studies of complement activation by *Leishmania* promastigotes have used either unfractionated or incompletely fractionated parasites to examine the complement pathway being activated or the extent of C3 deposition. Pearson and Steigbigel (25) concluded that *L. donovani* promastigotes activated the classical pathway in an antibody-dependent manner. Although subsequent studies (11) have arrived at the same conclusion, other workers (26, Puentes, S. M., et al., manuscript in preparation) suggested that complement activation occurs via the alternative pathway by these forms. In contrast, alternative pathway activation by unfractionated *L. braziliensis panamensis*, *L. donovani*, *L. major*, and *L. mexicana amazonensis* has consistently been reported (11, 27). Our results indicate that any simple categorization of the complement pathway being activated by a particular *Leishmania* species is likely to be inconclusive since different growth phases of the same strain and species can activate different pathways.

We observed a C3 band of  $110 \times 10^3$  mol wt on PNA<sup>-</sup> promastigotes but not on LOG promastigotes before methylamine treatment. This band, which comigrates with the native  $\alpha'$   $110 \times 10^3$  mol wt fragment of C3b, suggests that this portion of the C3 is not attached covalently to a parasite acceptor molecule. However, alternative explanations need to be considered. First, the ester linkage between C3b and acceptor may be less stable on PNA<sup>-</sup> than on LOG, resulting in spontaneous cleavage of the C3b-acceptor bond during sample processing (28). Prompt boiling of samples in SDS sample buffer, as we have done, should minimize this possibility. Secondly, C3b may be covalently bound to a low molecular weight acceptor, such as a phospholipid, which does not appreciably alter migration of the  $\alpha'$  chain by SDS-PAGE. Lastly the  $110 \times 10^3$  mol wt band in PNA<sup>-</sup> could represent the  $68 \times 10^3$  mol wt fragment of iC3b attached by a methylamine-resistant linkage to a parasite constituent of  $\sim 40 \times 10^3$  mol wt; this seems unlikely however since no change in the intensity of the  $110 \times 10^3$  mol wt band occurred in the C3c release assay (not shown). Thus, the possibility remains that C3b has an affinity for the surface of metacyclic promastigotes, presumably via the modified form of the LPG, as if these molecules served as C3 receptors. Experiments are underway in our laboratory to examine this possibility.

Once C3 has bound to the surface of the *L. major* promastigote, it has one of two fates. It either remains associated with the surface of the parasite or is released spontaneously from the surface of the parasite by a cleavage event. This latter process may be mediated through an endogenous parasite protease that cleaves and releases fragments of C3 from the C3-acceptor. The rate of release of C3 fragments is nearly threefold greater for LOG promastigotes than for PNA<sup>-</sup> promastigotes and may be a reflection of the overall metabolic state of the LOG promastigotes. The release of proteolytically cleaved C3 fragments was seen in a similar series of experiments using *L. donovani* promastigotes (Puentes, S. M., et al., manuscript in preparation). Since Etges et al. (29) has shown that the major surface glycoprotein gp63 has protease activity, we are currently examining whether this is the protease responsible for C3 release.

We show here that C3 binds to the excreted factor expressed on the outer surface of both LOG and PNA<sup>-</sup> promastigotes. The importance of the LPG as a cell attachment antigen has been previously reported by Handman et al. (7-9) and by Sacks and da Silva (10). The relationship between the C3 binding and cell attachment functions of the LPG requires further definition. Of interest, Russell (30) has shown that gp63 is a major C3 acceptor to *L. mexicana*, and also serves as a cell attachment antigen. While our results do not argue that the *L. major* LPG is the only acceptor site for C3, quantitative analysis suggests that the LPG is certainly the predominant acceptor molecule.

The LPG produced by PNA<sup>-</sup> parasites is developmentally regulated. Sacks and da Silva (10) have suggested that the addition of a terminal sialic acid on PNA<sup>-</sup> LPG alters its migration on SDS-PAGE, enhances labeling of the LPG by periodate oxidation and borohydride reduction under conditions in which primarily sialic acid is labeled, and blocks the capacity of PNA to bind to subterminal galactose residues. The relationship of these biochemical modifications to the form of bound C3 and to the release of C3 from the parasite surface is not clear. In erythrocytes, the presence of sialic acid is often associated with conversion of

C3b to iC3b, an effect we did not see here (30). Nonetheless, it is intriguing that PNA<sup>-</sup> promastigotes do not activate the alternative complement pathway (Fig. 5), since sialic acid is known to inhibit alternative pathway activation in other systems (31).

It now appears that the complement system interacts with different species and growth phases of *Leishmania* in quite distinctive manners. These differences may profoundly influence the macrophage subpopulations and cellular receptors with which these different species interact. Blackwell et al. (14) and Mosser and Edelson (16) have previously shown that the iC3b receptor on mononuclear cells, CR3, plays an important role in the attachment of the *L. donovani* and *L. major* promastigotes to the macrophage. Although we have found that nearly three-fourths of the C3 bound to *L. donovani* promastigotes is present as iC3b (Puentes, S. M., et al, manuscript in preparation), 80–85% of the C3 bound to *L. major* promastigotes is in the form C3b, which binds to CR1 but not CR3. Furthermore, opsonized *L. major* promastigotes bind more efficiently and are internalized more efficiently than unopsonized parasites to cultured mouse (26) or human macrophages (da Silva, R., manuscript in preparation); the binding and internalization of only the PNA<sup>-</sup> metacyclic can be significantly inhibited by an mAb to CR1 but not monoclonals to CR3 (da Silva, R., et al., manuscript in preparation). This suggests that CR1 is important for PNA<sup>-</sup> metacyclic binding and entry into the macrophage. These results, which correlate with the studies we have presented here, provide a framework for further study on the role of complement receptors for successful cell invasion by *L. major* promastigotes.

### Summary

The binding of complement by two developmentally distinct stages of *Leishmania major* has been studied. Noninfective log phase growth (LOG) promastigotes (serum sensitive) activate complement with deposition of covalently bound C3b onto the surface of the parasite. Infective, peanut agglutinin (PNA<sup>-</sup>) metacyclic stage promastigotes (serum resistant) also bear mainly C3b after incubation in serum, but a major portion of deposited C3 is present as a  $110 \times 10^3$  mol wt C3 fragment. Whereas deposition of C3b on LOG promastigotes is mediated through the alternative pathway, PNA<sup>-</sup> parasites are unable to activate the alternative pathway in nonimmune serum. C3 is released from the parasite surface by proteolytic cleavage, at a rate which is nearly threefold greater for LOG than for PNA<sup>-</sup> promastigotes. Immunoprecipitation experiments show that the developmentally regulated lipophosphoglycan is a major C3 acceptor on both LOG and PNA<sup>-</sup> parasites. These experiments, which are the first to compare the form and processing of complement on infective and noninfective promastigotes of *Leishmania*, provide a framework for further definition of the differential C3 receptor-dependent uptake and survival of these parasites within mononuclear phagocytes.

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