Leishmania amazonensis promastigotes evade complement killing by interfering with the late steps of the cascade

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SUMMARY

During their growth *in vitro*, promastigotes of *Leishmania amazonensis* undergo differentiation from complement-susceptible to complement-resistant forms. Here, we demonstrate that both forms bind comparable amounts of C3 on their surfaces, with the predominant molecule species being the haemolytically active C3b. Likewise, equivalent amounts of C9 are deposited on both forms of promastigotes. However, while C9-bearing complexes are exposed on the cell surface of resistant promastigotes, they are cryptic in the susceptible stage of the parasites. The membrane fraction of complement-resistant promastigote lysates has the ability to inhibit complement-mediated haemolysis, blocking C9, but not C3 deposition to complement-activating complexes. Moreover, the membrane fraction of complement-resistant promastigote lysates can inhibit the late steps of guinea-pig erythrocyte lysis much more efficiently than complement-susceptible ones. Our results indicate that *L. amazonensis* promastigotes evade complement killing by inhibiting the cytolytic pathway of the complement cascade.

Key words: *Leishmania amazonensis*, complement, C9, promastigotes.

INTRODUCTION

The activation of the complement system is an important early mechanism of host defence against microbial infections, and has been shown to play a critical role in the defence against several diseases caused by protozoan parasites. In the mammalian hosts, prior to penetration into macrophages, *Leishmania* are exposed to complement in the plasma. Complement can cause 2 main effects: either it kills the parasite, or it facilitates the entry of the promastigotes into macrophages if the reaction only reaches the C3 deposition step.

Promastigotes of *Leishmania* spp. activate complement by the alternative pathway when incubated with fresh non-immune sera (Mosser & Edelson, 1984; Barral-Netto *et al*. 1987). However, the non-infective and infective stages of promastigotes exhibit remarkable differences in their susceptibilities to complement-mediated lysis. The infective metacyclic promastigotes of *L. major*, *L. donovani*, *L. amazonensis* and *L. braziliensis* are significantly more resistant to complement killing, than the non-infective procyclic promastigotes (Barral-Netto *et al*. 1987; Howard, Sayers & Miles, 1987; Puentes *et al*. 1988; Kweider *et al*. 1989). The complement resistance of promastigotes also varies among *Leishmania* species (Moser & Edelson, 1984; Franke *et al*. 1985). Recently, we have observed that, during cultivation *in vitro*, promastigotes of the subgenus *Leishmania* differentiate from complement-sensitive to complement-resistant stages, whereas promastigotes of the subgenus *Viannia* remain susceptible to complement lysis during all stages of *in vitro* growth (Noronha *et al*. 1997). Although the mechanisms used by the metacyclic promastigotes of *L. donovani* and *L. major* to circumvent complement action have been thoroughly studied (Puentes *et al*. 1988, 1989, 1990), the strategy used by *L. amazonensis* infective promastigotes to evade complement is poorly understood.

Recently, we have demonstrated that natural antibodies present in human serum and proteases from the promastigotes, including gp63, do not have a role in *L. amazonensis* resistance to serum killing (Nunes & Ramalho-Pinto, 1996). Here, we report that, when complement is activated through the alternative pathway by *L. amazonensis* promastigotes, the deposition of the components C3b and C9 on the parasite surface is equivalent in both susceptible and resistant parasites. However, C9 is more exposed at the surface of susceptible parasites. We also show that the membrane fraction of complement-resistant stages of *L. amazonensis* promastigotes inhibits complement-dependent haemolysis and the deposition of C9 on human IgG-coated

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plastic, without interfering with the deposition of C3. Moreover, lysates of complement-resistant promastigotes are more efficient than complement-susceptible ones to inhibit the cytolytic steps.

MATERIALS AND METHODS

Parasite cultivation

L. amazonensis promastigotes strain IFLA/BR/67/PH8 were used throughout this work. Promastigotes were grown at 25 °C in RPMI-1640 containing 15 % (v/v) heat-inactivated FCS (Gibco-BRL, Grand Island, NY), 25 mM Hepes and 50 μg/ml gentamycin sulfate. Cultures were seeded by inoculation of late-logarithmic phase promastigotes to the starting density of $1{\cdot}5 \times 10^6$/ml and parasites were counted daily by using a haemocytometer. Complement-sensitive and complement-resistant promastigote forms were collected at early logarithmic phase (day 2; $4{\cdot}15 \pm 0{\cdot}79 \times 10^6$ parasites/ml) and early stationary phase (day 5; $2{\cdot}82 \pm 0{\cdot}41 \times 10^7$ parasites/ml), respectively, by low-speed centrifugation, washed in PBS, and used in all experiments.

Complement source

Fresh normal human sera (f-NHS) were collected from healthy volunteers with no history of previous exposure to *Leishmania* spp. Blood was allowed to clot for 30 min at room temperature, and left for 3 h at 4 °C. Sera were collected, aliquoted, frozen at −80 °C, and thawed immediately before use. For some experiments, sera were previously inactivated by heating at 56 °C for 30 min (hi-NHS).

Analysis of C3 and C9 binding to the surface of promastigotes

The amount of C3 and C9 bound to promastigotes incubated with f-NHS was determined by ELISA. Promastigotes were harvested by centrifugation and washed 3 times in ice-cold PBS. Parasites were resuspended in 10 mM sodium carbonate buffer (pH 9·6) to a concentration of 1×10^7 cells/ml. One hundred μl of this suspension were added to each well of a 96-well microtitre plate (Nunc Maxisorp, Denmark), and incubated overnight at 37 °C, when parasites adhered to the plastic. The plate was washed with 5 mM sodium barbital (pH 7·4), 140 mM NaCl, 1 mM $MgCl_2$, 0·15 mM $CaCl_2$ (VBS^{2+}) and incubated with different concentrations of f-NHS in VBS^{2+} at 37 °C for 45 min. In kinetic studies, the plate was incubated with 10 % (v/v) f-NHS in VBS^{2+} at 37 °C for various periods of time. The reactions were stopped with PBS containing 10 mM EDTA. The plates were rinsed with PBS–0·05 % (v/v) Tween 20 and then blocked with PBS plus 5 % (w/v) bovine serum albumin (BSA) for 1 h at room temperature. C3 and C9 were detected on the surface of parasites using a rabbit antiserum anti-C3c (Sigma) (1:40000), or a rabbit antiserum against C9 plus poly-C9 (a gift from Dr J. Ding-E. Young, Rockefeller University, NY) (1:2000), respectively. After incubation for 1 h, the wells were washed with PBS–0·05 % (v/v) Tween 20 and incubated with a peroxidase-conjugated anti-rabbit IgG (1:20000) for a further 1 h. Finally, 100 μl of *o*-phenylenediamine (OPD) and H_2O_2 solution were added and the reaction was stopped with 50 μl of 20 % sulfuric acid. The absorbance was measured at 492 nm in an automated ELISA microplate reader (Bio-Rad, Richmond, USA).

Determination of the form and shedding of the C3 bound to promastigote surface

To determine which fragment of C3 was bound to the parasite surface, we used the Gaither's C3c release assay (Gaither, Hammer & Frank, 1979). Promastigotes adhered to microtitre plates (2×10^6/well) were incubated in different dilutions of f-NHS in VBS^{2+} for 45 min at 37 °C. After washing with the same buffer, parasites were treated with TPCK-trypsin (Sigma) at 2·5 μg/ml for 10 min at 30 °C. The reaction was stopped with PBS–20 % (v/v) FCS, and the C3 fragments that remained bound on treated parasites were quantified by ELISA, as described above.

The shedding of the bound C3 was determined over a period of 90 min. The parasites were pre-treated with 10 % (v/v) f-NHS in VBS^{2+} for 45 min at 37 °C, washed twice in VBS^{2+}, resuspended in VBS^{2+} and incubated at 37 °C. At different time-points, the amount of C3 fragments that remained bound to parasites was quantified by ELISA, as described above.

Determination of the accessibility of C9 bound to promastigote surface

The accessibility of bound C9 to specific antibodies was measured after digestion of serum-treated parasites with trypsin. f-NHS or hi-NHS-treated organisms (5×10^7 cells/ml in 10 % (v/v) f-NHS for 45 min at 37 °C) were resuspended to the same density in 100 μg/ml TPCK-trypsin in VBS^{2+}, and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 2-fold excess soy bean trypsin inhibitor (SBTI), and the C9 that remained bound to parasites was quantified by ELISA, as described above.

Preparation of promastigote lysates

L. amazonensis promastigotes harvested from culture were washed in PBS and then centrifuged at 100 ***g*** for 10 min. The pellet was resuspended to a density

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of 5×10^9 parasites/ml in 20 mM Tris–HCl (pH 8·0) containing a cocktail of protease inhibitors comprised of 1 mM PMSF, 1 mM 1,10-phenanthroline, 0·1 mM E-64, 10 μg/ml pepstatin A. Parasites were disrupted by 5 cycles of freeze-and-thaw and centrifuged at 100000 ***g*** for 1 h at 4 °C. The supernatant was recovered and kept on ice, and the pellet washed and resuspended to the original volume. Protein concentration was determined as previously described (Bradford, 1976) using the Bio-Rad reagent kit according to the manufacturer's instructions.

Haemolytic assays

Sheep (S), rabbit (Rb) or guinea-pig (Gp) red blood cells (RBC), kept in Alsever's solution at 4 °C for no more than 3 weeks, were washed 3 times with PBS before use. For haemolytic assays, 5×10^6 SRBC/well of U-bottomed microtitre plates (Nunc) were incubated with increasing concentrations of f-NHS acidified to pH 6·4 with 0·1 M HCl at 37 °C for 30 min in 250 μl of VBS^{2+} containing 0·1 % gelatin (w/v) ($GVBS^{2+}$). Alternatively, RbRBC were incubated with f-NHS under the same conditions. In reactive lysis assays, GpRBC were incubated at 37 °C for 30 min in 5 % (v/v) EDTA-chelated NHS containing 5 % (v/v) pre-assembled Cobra Venom Factor (CVF)-Bb convertases (100 ng of CVF purified according Beukelmann *et al*. (1987), 1 % (v/v) f-NHS in GVBS containing 2 mM $MgCl_2$ and 8 mM EGTA, at 37 °C for 30 min). After incubation, the plate was centrifuged at 200 ***g*** for 10 min, and the amount of released haemoglobin was determined at 414 nm in an automated ELISA microplate reader. The effect of *L. amazonensis* promastigote lysates on the activation of the alternative pathway of human complement was assayed by adding 25 μl of either the supernatant or the resuspended pellet of parasite lysates to the wells containing erythrocytes plus serum.

Complement deposition on IgG-adsorbed plastic

ELISA assays were used for the detection of complement activation products (Zwirner *et al*. 1989). Human IgG was affinity purified from NHS using protein G-Sepharose (Pharmacia LKB, Uppsala, Sweden). A solution of 10 μg/ml of purified human IgG in sodium carbonate buffer (pH 8·2) was added to 96-well microtitre plastic plates (Nunc) and incubated overnight at 37 °C. The plates were blocked with 5 % (w/v) BSA in PBS for 1 h, and then incubated with 10 % (v/v) f-NHS in VBS^{2+} for 45 min at 37 °C, in the presence or absence of a 2-fold serial dilution of the serum-resistant promastigote lysates. After extensive washes with PBS–0·05 % (v/v) Tween 20, the plates were incubated for 1 h with either anti-human C3c IgG (1:20000) or anti-human C9 plus poly-C9 antisera (1:2000). The plates were then incubated with peroxidase-conjugated anti-rabbit IgG (1:20000) for 1 h and developed with the peroxidase substrate OPD and H_2O_2.

Statistical analysis

All experiments were performed at least 3 times. Statistical significance ($P < 0{\cdot}05$) was determined by Student's *t*-test.

RESULTS

C3 binding to and release from complement-sensitive and -resistant promastigotes

Typical growth curves of promastigotes of *L. amazonensis* cultured *in vitro* showed a lag phase of 24 h, a logarithmic phase from day 1 to day 4 and a stationary phase from day 5 onwards (data not shown). From day 1 to day 3 (early-logarithmic), promastigotes were susceptible to lysis by the complement system. Approaching the late-logarithmic and early stationary phases (days 4 and 5), parasites developed a resistance to complement lysis that decreased thereafter to the original susceptibility after 6 days of culture (Noronha *et al*. 1997).

To investigate whether the complement resistance of *L. amazonensis* promastigotes was related to different levels of C3 deposition onto the parasite surface, we measured the binding of C3 to complement-sensitive (day 2) and complement-resistant (day 5) promastigotes previously incubated with different concentrations of f-NHS at 37 °C for different periods of time. We found that the 2 stages of cultured promastigotes bound equivalent amounts of C3 to their surfaces (Fig. 1A), with similar kinetics (Fig. 1B), maximal binding occurring at 10 min of incubation in f-NHS.

Analysis of the form and the stability of the C3 bound to promastigote surface

To estimate the proportion of C3b or iC3b bound to the surface of the complement-resistant and complement-sensitive promastigotes, we have used an adaptation of the Gaither's C3c release assay described in the Materials and Methods section. This assay is based on the fact that cell-bound iC3b, but not C3b, is susceptible to cleavage by low doses of trypsin, releasing the C3c fragment into the supernatant. Susceptible or resistant promastigotes were treated with f-NHS and then with low doses of trypsin. Analysis of the remaining C3 bound to the parasite surface (Fig. 2) showed that both stages of promastigotes released equal amounts of C3 (less than 30 %), corresponding to the inactive form iC3b. This indicates that in both sensitive and resistant forms of *L. amazonensis*, more than 70 % of the bound C3 is the haemolytically active C3b. Moreover, less than 10 % of parasite-bound C3 fragments

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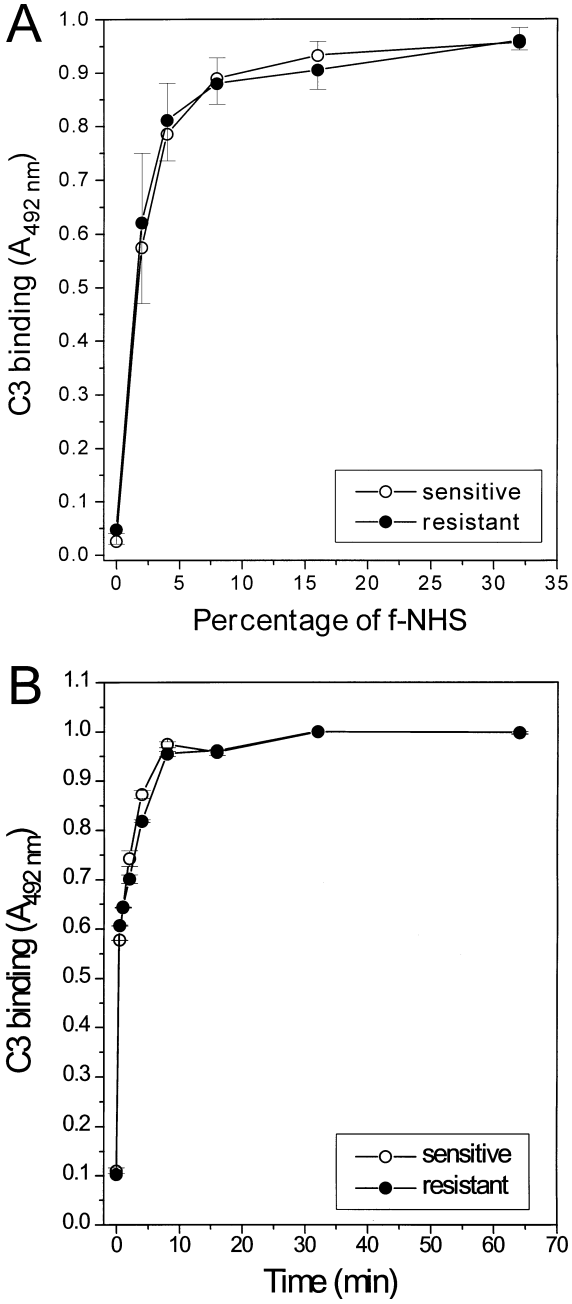

Fig. 1. C3 binding to *Leishmania amazonensis* promastigotes. Complement-sensitive (day 2) and -resistant (day 5) promastigotes were incubated with different concentrations of f-NHS at 37 °C for 45 min (A) or in 10% f-NHS at 37 °C for increasing times (B). C3 was quantified by ELISA using an anti-human C3c antiserum.

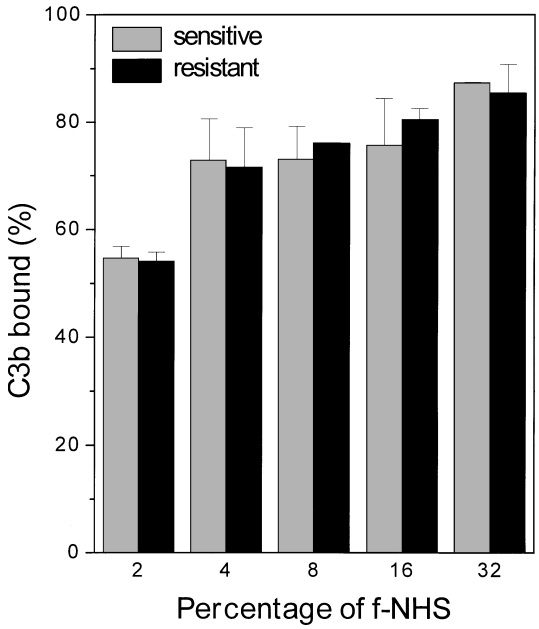

Fig. 2. Form of C3 fragments bound to *Leishmania amazonensis* promastigotes. Complement-sensitive (day 2) and -resistant (day 5) promastigotes were incubated with different concentrations of f-NHS at 37 °C for 45 min. Complement-treated parasites were incubated with 2·5 μg/ml of TPCK-trypsin for 10 min at 30 °C. C3 that remained bound to parasites, corresponding to C3b, was quantified by ELISA using an anti-human C3c antiserum.

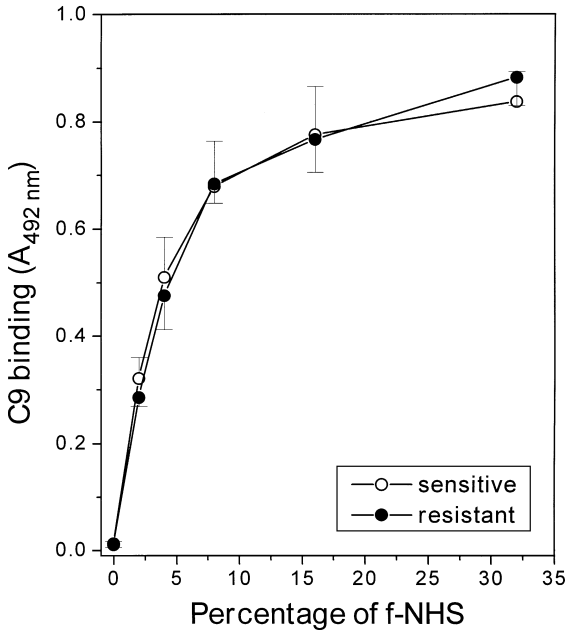

Fig. 3. C9 binding to *Leishmania amazonensis* promastigotes. Complement-sensitive (day 2) and -resistant (day 5) promastigotes were incubated with different concentrations of f-NHS at 37 °C for 45 min. C9 was quantified by ELISA using an anti-human C9 plus poly-C9 antiserum.

were spontaneously released from the surfaces of serum-treated parasites even after 90 min of incubation at 37 °C, irrespective of their complement sensitivity, indicating that the C3b was not shed (data not shown).

C9 binding to complement-sensitive and complement-resistant promastigotes

The amount of C9 bound to promastigotes following incubation with f-NHS, was measured in either complement-sensitive logarithmic promastigotes (day 2), or complement-resistant stationary promastigotes (day 5). Figure 3 shows that both stages of

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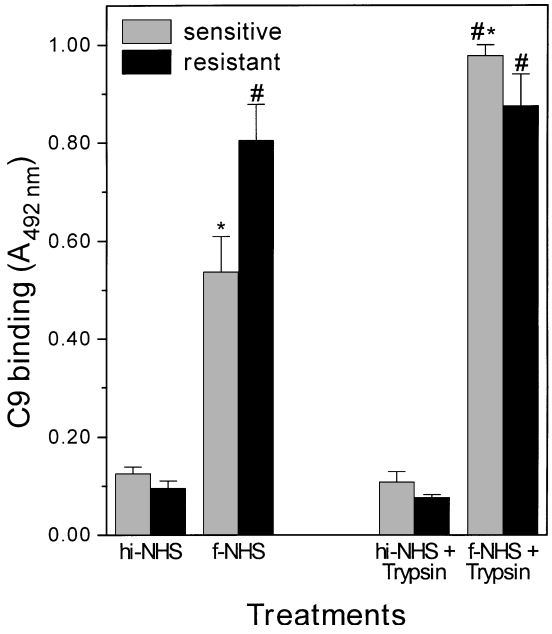

Fig. 4. Accessibility of C9 antibodies to membrane attack complex (MAC) assembled in *Leishmania amazonensis* promastigotes. Complement-sensitive and -resistant promastigotes were incubated in 10% (v/v) f-NHS or hi-NHS at 37 °C for 45 min. Serum-treated parasites were incubated with 100 μg/ml of TPCK-trypsin for 15 min at 37 °C, and C9 bound to the parasite surface was quantified by ELISA using an antiserum against human C9 plus poly-C9. Data correspond to the mean ± S.E. of 3 experiments. #, not significant; *, statistically significant, $P < 0{\cdot}05$.

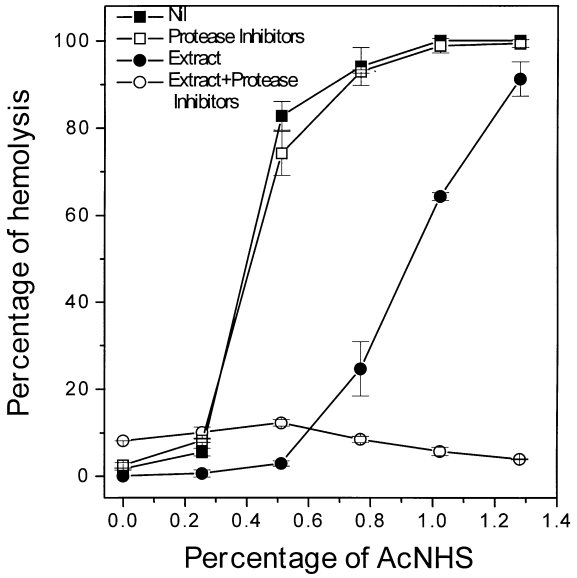

Fig. 5. Inhibition of SRBC haemolysis, mediated by acidified f-NHS (AcNHS), by *Leishmania amazonensis* complement-resistant promastigote lysates. SRBC (5×10^6/well) were incubated with increasing concentrations of AcNHS in $GVBS^{2+}$ containing 50 μl of either buffer (□, ■) or promastigote lysates (○, ●) in the presence (open symbols) or absence (closed symbols) of protease inhibitors, as specified in the Materials and Methods section, at 37 °C for 30 min. Lysis was evaluated by the absorbance of the supernatant at 414 nm. Haemolysis is represented as the percentage of total lysis obtained by incubation of SRBC with water.

promastigotes bound similar amounts of C9 to their surfaces. In live parasites, not fixed to plastic microplates, the amount of C9 attached to their surface was significantly higher in resistant than in sensitive promastigotes (Fig. 4). However, when NHS-incubated promastigotes are treated with trypsin, equivalent amounts of C9 were detected by anti-C9-specific antibodies on the surface of parasites at both stages of development (Fig. 4). This result suggests that, in complement-sensitive promastigotes, C9 molecules are cryptic in their membranes while they are exposed on the surface of complement-resistant parasites, being accessible to antibodies.

Complement inhibitory activity of serum-resistant promastigote lysates

To investigate whether parasite components could interfere with the alternative pathway activation of f-NHS, 2 haemolytic systems were used. In the first, we used f-NHS acidified to pH 6·4, known to activate the alternative pathway of human complement and lyse SRBC (Fishelson, Horstmann & Muller-Eberhard, 1979). In the second, we used RbRBC, which are capable of directly activating the alternative pathway of human complement (Platts-Mills & Ishizaka, 1974). We have found that resistant promastigote lysates prepared in the presence of protease inhibitors totally inhibited SRBC haemolysis induced by acidified f-NHS (Fig. 5). However, the lack of protease inhibitors during the preparation of the lysates greatly reduced their inhibitory activity. This indicates that the promastigote complement-inhibitory activity is associated with protease-sensitive polypeptides of the parasite lysates.

To investigate where this complement-inhibitory protein is localized, we centrifuged the parasite lysates at 100000 ***g*** for 1 h, and assayed both pellet and supernatant for their ability to block complement-mediated haemolysis. Figure 6A shows that the membrane-containing pellet completely blocked SRBC lysis caused by acidified f-NHS, whereas the supernatant was unable to inhibit haemolysis. Similar results were obtained for the RbRBC/f-NHS lytic assay, in which the promastigote membrane-rich fraction, but not the soluble supernatant, markedly inhibited complement-mediated haemolysis (Fig. 6B).

Blocking of C9 deposition by serum-resistant promastigote lysates

To determine at which step of the complement cascade the membrane-associated inhibitory protein

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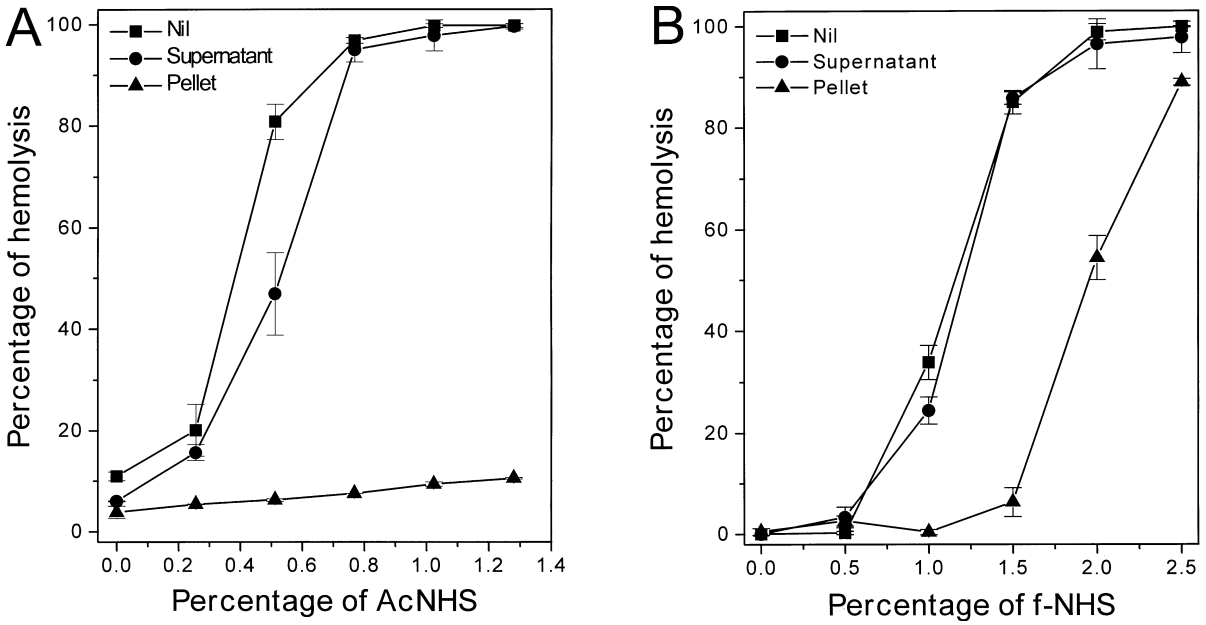

Fig. 6. Inhibition of SRBC haemolysis, mediated by acidified f-NHS (AcNHS), or RbRBC haemolysis, mediated by f-NHS, by *Leishmania amazonensis* complement-resistant promastigote lysate fractions. (A) SRBC or (B) RbRBC (5×10^6/well) were incubated with increasing volumes of AcNHS or f-NHS, respectively, in $GVBS^{2+}$ containing 50 μl of either supernatant (●) or pellet (▲) from ultracentrifuged lysates of 5×10^9 complement-resistant (day 5) promastigotes, or buffer alone (■), at 37 °C for 30 min. Lysis was evaluated by the absorbance of the supernatant at 414 nm. Haemolysis is represented as the percentage of total lysis obtained by incubation of RBC with water.

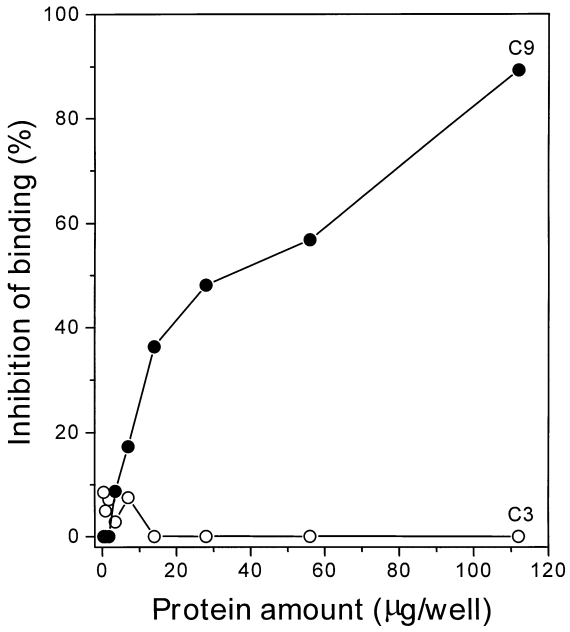

Fig. 7. Inhibition of C3 and C9 binding to adsorbed human IgG by *Leishmania amazonensis* complement-resistant promastigote lysates. Complement activation was achieved by incubating the IgG-coated wells with 10% f-NHS in VBS^{2+} containing 1% BSA for 45 min at 37 °C in the presence or absence of different amounts of complement-resistant (day 5) promastigote lysates. The amount of C3 and C9 was determined by ELISA using anti-human C3 or anti-human C9 plus poly-C9 antiserum. Inhibition is calculated in relation to the binding of complement components in the absence of promastigote lysates.

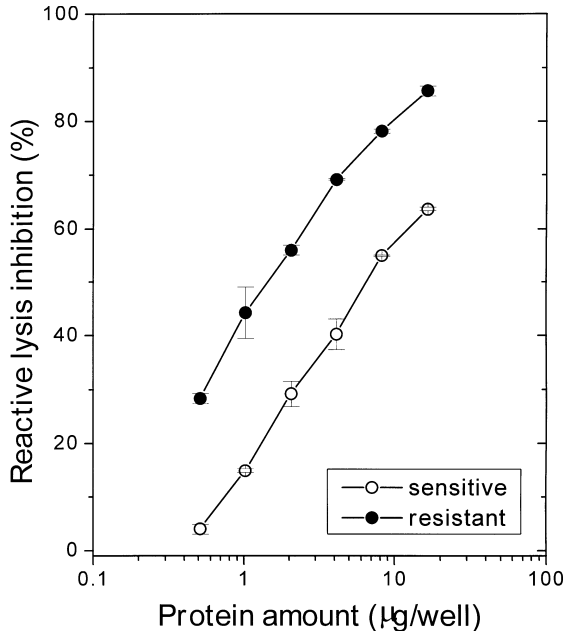

Fig. 8. Inhibition of reactive lysis by *Leishmania amazonensis* complement-sensitive or -resistant promastigote lysates. GpRBC (1×10^7/well) were incubated with pre-assembled CVF-Bb convertases in 45% EDTA-chelated f-NHS, in the presence of different amounts of complement-sensitive (day 2) and -resistant (day 5) promastigote lysates at 37 °C for 30 min. Lysis was evaluated by the absorbance of the supernatant at 414 nm. Inhibition is calculated in relation to lysis in the absence of promastigote lysates.

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was acting, we used a complement-activating system consisting of human IgG adsorbed to polystyrene microtitre plates, known to activate complement through the classical pathway. As shown in Fig. 7, the lysates of complement-resistant promastigotes did not interfere with the binding of C3 to adsorbed IgG. However, it inhibited the binding of C9 to the plastic substrate, in a dose-dependent fashion, reaching more than 90% inhibition with about 100 μg of protein. This result indicates that the interference of *L. amazonensis* promastigotes with the complement cascade occurs at a level further than the C3b generation, possibly at the stage of C9 deposition.

The blocking of the cytolytic pathway was verified by using a haemolytic assay known as reactive lysis, in which functional pore formation is dependent on fluid phase C3/C5 convertases made of CVF instead of human serum C3. As shown in Fig. 8, lysates of serum-resistant and also serum-susceptible promastigote stages are able to block reactive lysis in a dose-dependent manner. However, about 5 times less lysate of serum-resistant promastigotes is necessary to achieve the same inhibition level than the lysate of serum-susceptible promastigotes.

DISCUSSION

Parasites have developed a variety of mechanisms to escape complement damage. These include the blocking of the initiation of the complement cascade, the proteolytic degradation of complement proteins deposited on the parasite surface, and the release of complement components bound to acceptor molecules of the parasite surface (Fishelson, 1991).

Leishmania spp. display an arsenal of strategies to escape from complement lysis when in contact with serum. *L. donovani* promastigotes block the complement cascade either by proteolytic degradation of deposited C3b to iC3b or by active release of parasite-bound C3b (Puentes *et al*. 1989). *L. major* metacyclic promastigotes efficiently activate the complement cascade, and C3 and C5b-7 deposition are similar in both resistant and susceptible forms. However, in metacyclic promastigotes the MAC is not correctly inserted into the lipid bilayer and is subsequently released to the environment (Puentes *et al*. 1988, 1990). In *L. amazonensis* promastigotes transfected with a *L. major* gp63 gene, the presence of the proteolytically active gp63 correlates with an accelerated conversion of C3b to an inactive form, resulting in an increased resistance to complement-mediated lysis (Brittingham *et al*. 1995). However, we have not detected any increase in the activity (Nunes & Ramalho-Pinto, 1996) or protein expression of gp63 during the transition from susceptible to resistant forms of promastigotes, in SDS–PAGE, followed either by silver staining (data not shown) or by immunoblotting using monoclonal antibody anti-gp63 (Nunes & Ramalho-Pinto, 1996). It is possible, therefore, that the artificial overexpression in gp63-transfected promastigotes could be responsible for the increased C3b inactivation.

L. amazonensis promastigotes are able to activate complement through the alternative pathway (Mosser & Edelson, 1984; Soares *et al*. 1993). Recently, we have observed that *in vitro*-cultured *L. amazonensis* promastigotes from all growth phases can be killed by f-NHS in a dose-dependent fashion, although their susceptibility to complement-mediated lysis varies as they develop. On days 1 and 2 (early-logarithmic phase) promastigotes are extremely susceptible to f-NHS. However, they develop a resistance that peaks on days 4 and 5 (late-logarithmic and early-stationary phases) decreasing thereafter up to day 8 (late-stationary phase), when parasites are again as susceptible as they were in early-logarithmic phase (Noronha *et al*. 1997).

Using parasites from the early logarithmic and the early stationary phases, we have demonstrated that the serum-resistant stages of *L. amazonensis* promastigotes bind C3 fragments to the same extent and with similar kinetics as the serum-susceptible forms, when incubated with f-NHS. In this aspect, *L. amazonensis* promastigotes resemble *L. donovani* promastigotes (Puentes *et al*. 1989) but, unlike these and *L. major* promastigotes (Puentes *et al*. 1988), they do not spontaneously shed C3 from their surface. The spontaneous release of C3 bound to the membrane of f-NHS-pre-treated *L. amazonensis* promastigotes in serum-free buffer is minimal, even after 90 min at 37 °C (data not shown). The predominant form of C3 on the parasite surface is the haemolytically active C3b, since more than 70% of the C3 deposited on *L. amazonensis* promastigotes is resistant to trypsin treatment (Gaither *et al*. 1979). In this respect, *L. amazonensis* promastigotes resemble *L. major* (Puentes *et al*. 1988) and *L. mexicana* promastigotes (Russell, 1987). These results show that resistance to complement-mediated lysis of early stationary phase-promastigotes is not due to an impaired C3b binding or proteolytic inactivation on the parasite surface, again in contrast with the findings that gp63 impairs C3b surface deposition (Brittingham *et al*. 1995).

Structural modifications in membrane components have been described during the process of transformation of promastigotes from the non-infective to the infective stage, either in the gut of sandflies (Sacks & Perkins, 1985) or during cultivation *in vitro* (Sacks & da Silva, 1987). The most remarkable of these surface changes are the elongation of LPG molecules (McConville *et al*. 1992) and the thickening of the glycocalyx (Pimenta, Saraiva & Sacks, 1991) in *L. major* promastigotes. These changes have been implicated in the complement resistance of *L. major* metacyclic promastigotes that

cause C5b-9 complexes to bind to these surface components, far from the parasite membrane (Puentes *et al*. 1990). From our observations, a similar mechanism could, at least in part, contribute to the escape of *L. amazonensis* promastigotes. Apparently, in serum-resistant promastigotes, C9-bearing complexes are superficially attached to the parasite, readily available for recognition by anti-C9 antibodies. On the contrary, in serum-susceptible parasites these complexes seem to be buried in the membrane, being recognized by anti-C9 antibodies only after mild trypsinization of the parasite surface.

We have also found that serum-resistant promastigotes contain membrane-associated proteins, capable of blocking the lytic activity of complement on RBC membranes, after activation of the alternative pathway. These putative inhibitory proteins impair C9, but not C3 binding to complement-activating complexes, indicating that complement blocking occurs at later steps of the complement cascade, after the C3b deposition, probably at the stage of C9 deposition. The action of promastigote inhibitor at the cytolytic pathway was corroborated by reactive lysis findings. In addition, these results suggested that complement resistance could be related to different quantities of inhibitor expressed on the surface of complement-sensitive and -resistant parasites.

We have observed that promastigotes in culture display an enhanced expression of 3 polypeptides that parallels the development of the parasite's complement resistance (Noronha *et al*. 1997) and the expression of the complement inhibitory activity. It is possible that 1 or more of these molecules may be involved in these processes. An abundant surface glycoprotein of promastigotes of *L. amazonensis* is the gp46 (McMahon-Pratt & David, 1982). Interestingly, gp46 presents 2 well-defined structural domains, one of which is a 27 kDa polypeptide generated by poteolytic cleavage of the intact protein that is very resistant to further proteolysis (Kahl & McMahon-Pratt, 1987). Coincidently, 46 kDa and 27 kDa are the molecular masses of 2 out of the 3 polypeptides whose expression is increased in complement-resistant parasites. So far, no function has been ascribed for gp46. Since the *L. braziliensis* complex species do not express gp46 (McMahon-Pratt *et al*. 1992) and they do not differentiate in complement-resistant forms (Noronha *et al*. 1997), it is plausible to speculate that members of the gp46 family could be responsible for the complement inhibitory activity. We are currently investigating this hypothesis, and preliminary results corroborate this hypothesis.

Taken together, our results indicate that the resistance to complement lysis of *L. amazonensis* promastigotes might be, at least in part, due to the impairment of C9 deposition on the parasite surface. It is possible that the putative complement inhibitory protein(s) acts by preventing the correct insertion of C9 and/or MAC functional assembly in the parasite lipid bilayer.

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