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Cytolytic Activity in the Genus *Leishmania*: Involvement of a Putative Pore-Forming Protein

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We describe here that parasites of the genus *Leishmania* contain a cytolytic activity which acts optimally at pH 5.0 to 5.5 and at 37°C in vitro. Of the four species examined, *Leishmania* (*Leishmania*) *amazonensis* and *Leishmania* (*Leishmania*) *major* presented considerable hemolytic activity, whereas *Leishmania* (*Viannia*) *panamensis* and *Leishmania* (*Viannia*) *guyanensis* showed little and no hemolytic activity, respectively. The cytolytic factor of *L. amazonensis* promastigotes was characterized as a protein with no protease-, phospholipase-, or detergent-like activity, probably localized inside membranous vesicles. The use of osmotic protectants revealed the colloid-osmotic nature of hemolysis, which is indicative of pore formation in the membranes of target cells. This putative pore-forming protein also damaged nucleated cells, including macrophages, causing an increase in their membrane permeability with leakage of cytoplasmic proteins. Both promastigotes and amastigotes express this lytic activity, suggesting that the cytolysin may have a function in both stages of this parasite. The pH and temperature required for optimal activity indicate that it might be more effective within the mammalian host, particularly inside the macrophage parasitophorous vacuole. In promastigotes of *L. amazonensis*, the expression of lytic activity seems to be regulated during their growth in vitro, being maximal at the early stationary phase.

Leishmania spp. are the causative agents of a disease that manifests itself in humans in a variety of clinical forms, depending upon the species involved and the immunological status of the host (25). These include cutaneous, mucocutaneous, and visceral leishmaniasis; the last is often fatal if not successfully treated. In vertebrates, these protozoans are obligatory intracellular parasites (see references 13 and 26 for reviews). They invade macrophages and replicate in parasitophorous vacuoles, which are membranous structures filled with acidic fluid that surround the parasite during the invasion process. The first invading forms are promastigotes, transmitted to vertebrates by bloodsucking sandflies. Promastigotes are long flagellated forms that develop extracellularly inside the digestive tracts of insects. Once inside the parasitophorous vacuole, promastigotes transform into amastigotes, which are round nonmotile forms that multiply rapidly. Although the interaction between parasite and macrophage appears to be a lasting relationship (42), the parasitized cell eventually bursts (13, 26). The released amastigotes can then enter neighboring healthy macrophages and repeat this cycle.

The bursting of macrophages, a key step in the spreading of these parasites, is an event whose mechanism is completely unknown. However, it is apparent that damage of the parasitophorous vacuole and plasma membranes is a prerequisite for cell rupture. Recently, we have reported that *Leishmania amazonensis* has a hemolysin active at acid pH which might be involved in the rupture of the macrophage (29). Here, we further characterize this lytic molecule, presenting evidence which indicates that it is a pore-forming protein (PFP) probably localized inside parasite membranous vesicles. We show that this putative PFP is also found in amastigotes and is active against nucleated cells. We also show that this lytic activity is present in another strain of *L. amazonensis* and in another species of the subgenus *Leishmania*, *L. major*, but this is not a feature of all members of the genus *Leishmania*, since it is poorly expressed or completely absent in two species of the subgenus *Viannia.*

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MATERIALS AND METHODS

Parasites. The PH8 (IFLA/PA/67/PH8) strain of *Leishmania* (*Leishmania*) *amazonensis*, used throughout this work, and the M1176 (MHOM/BR/70/M1176) strain of *Leishmania* (*Viannia*) *guyanensis* were provided by Maria Norma Melo (Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil). Strain LTB0016 (MHOM/BR/77/LTB0016) of *Leishmania* (*Leishmania*) *amazonensis*, WR309 (MHOM/IL/79/LRC-L251) of *Leishmania* (*Leishmania*) *major*, and *Leishmania* (*Viannia*) *panamensis* were provided by Diane McMahon-Pratt (Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Conn.). Promastigotes were obtained from axenic culture at 23°C in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (Sigma), 15% heat-inactivated fetal bovine serum (GIBCO, Grand Island, N.Y.), and 50 μg of gentamicin (Sigma) per ml. Four-day-cultured parasites were washed three times with phosphate-buffered saline (PBS), pelleted by centrifugation at 1,000 × *g*, and kept at −70°C until required. Amastigotes were obtained from lesions of 8-week-old BALB/c mice that had been inoculated into the hind footpads with 5×10^5 promastigotes. Two months later, the lesions developed in the mouse footpads were excised, and the infected tissues were completely broken up in culture medium to free the amastigotes. The cell suspension was centrifuged at 200 × *g* to remove cell debris and then at 1,000 × *g* to sediment amastigotes. Parasites were extensively washed with PBS before the preparation of extracts. For practical purposes, we used promastigotes throughout this study, unless otherwise stated.

Parasite extracts. Promastigotes or amastigotes were resuspended in 10 mM Tris-HCl (pH 7.5) to a density of 2×10^6 parasites per μl and subjected to five cycles of freeze-thaw. The extract was centrifuged at 1,000 × *g* for 10 min at 4°C to sediment intact cells and nuclei, and the supernatant, referred to as crude extract (cExt), was used in most experiments. For some experiments, the cExt was centrifuged at 100,000 × *g* for 1 h at 4°C. The membrane-enriched pellet was resuspended to the original volume with 10 mM Tris-HCl (pH 7.5) containing 0.6% CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} (Sigma) and kept for 30 min on ice, with occasional agitation. This was centrifuged at 100,000 × *g*, and the supernatant was referred to as soluble extract (sExt). cExt and sExt contained approximately 2 and 1.3 mg of protein per ml, respectively. Alternatively, the membrane-enriched pellet was treated with 5 mM

TABLE 1. Hemolytic activities of promastigotes under different conditions of incubation and effects of metabolic inhibitors and calcium ionophores

Medium	pH/temperature (°C)	Treatment	% Hemolysis[a] ± SD	% Dead parasites ± SD	
				2 h	6 h
Acetate	5.5/37	None	81.0 ± 4.0	30.0 ± 4.0	98.0 ± 1.0
RPMI	5.5/37	None	35.0 ± 12.0	20.0 ± 3.0	70.0 ± 6.0
RPMI	5.5/37	5 mM sodium azide	90.0 ± 2.0	100.0 ± 0.0	100.0 ± 0.0
RPMI	5.5/37	50 mM 2-deoxy-D-glucose	81.0 ± 4.0	56.0 ± 9.0	90.0 ± 6.0
RPMI	7.0/25	None	12.0 ± 3.0	6.0 ± 2.0	8.0 ± 2.0
RPMI	7.0/25	2 μg of A23187 ml^{-1}	16.0 ± 1.0	6.0 ± 1.0	45.0 ± 9.0
RPMI	7.0/25	2 μM ionomicin	19.0 ± 4.0	27.0 ± 2.0	34.0 ± 8.0

[a] At 6 h.

EDTA for 1 h at 25°C to extract peripheral membrane proteins (18, 39) or with 100 mM Na_2CO_3 for 30 min at 0°C to open membranous vesicles (18). The extracts were centrifuged at 100,000 × *g*, and both supernatant and pellet were assayed for hemolytic activity, after acidification to pH 5.5, at a 1:20 final dilution in microplates with 96 round-bottomed wells, as described below. In these cases, mock-treated controls were included with 100 mM Na_2CO_3 or 5 mM EDTA, acidified to the same pH, and used at the same dilution as was the membrane extract.

Hemolytic assays. We used a protocol modified from Andrews and Whitlow (5). In Eppendorf tubes, 2×10^7 parasites were incubated for different periods in 200 μl of lysis buffer (10 mM acetate buffer, 150 mM NaCl, pH 5.5) or in RPMI 1640 (pH 5.5 or 7.0) at 25 or 37°C. Parasites were then incubated with 5×10^6 human erythrocytes (HuRBC) at 37°C for 30 min at pH 5.5. Some experiments were carried out in the presence of 5 mM sodium azide, 50 mM 2-deoxy-D-glucose, 2 μg of A23187 per ml, or 2 μM ionomicin. Parasite extracts were assayed in microplates with 96 round-bottomed wells on which 5×10^6 HuRBC, sheep RBC, or rabbit RBC in 200 μl of lysis buffer were incubated with 10 μl of serially diluted extracts of 2×10^6 parasites per μl. After 30 min at 37°C, each microplate was centrifuged for 10 min at 500 × *g* and lysis was quantitated by the hemoglobin released, as determined by reading the A_{414} of the supernatant. The percentage of lysis was determined in relation to total lysis, obtained by incubation of the same number of RBC with 10 μl of 0.5% Triton X-100. To determine the optimal pH for the hemolytic activity, assays were carried out at different pHs in the range of 4.5 to 8.0. The following buffers, containing 150 mM NaCl, were used: at pH 4.5 to 5.5, 10 mM acetate buffer; at pH 6.0 and 7.0, 10 mM phosphate buffer; at pH 8.0, 10 mM Tris-HCl buffer. For some experiments, hemolytic activity was reported as the inverse of the dilution that caused 50% of hemolysis (H_{50}), as calculated by linear regression of percentage of lysis versus dilution curves. For optimal-temperature determination, assays were carried out in Eppendorf tubes incubated in water baths at 0, 15, 25, and 37°C.

Some experiments were carried out in the presence of 30 mM raffinose or 30 mM polyethylene glycol (PEG) 6000; others were carried out in the presence of protease inhibitors {5 mM *o*-phenanthroline, 1 mM phenylmethylsulfonyl fluoride (PMSF), or 0.1 mM E-64 [trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane] (Sigma)}, divalent cations (1 to 5 mM $CaCl_2$, $MgCl_2$, or $ZnSO_4$), cation chelators (10 mM EDTA or EGTA), or Rosenthal's inhibitor of phospholipase A_2 (dimethyl-DL-2,3-distearoyl-oxypropyl-2′-hydroxyethyl-ammonium acetate) (Sigma) (19, 41) at the final concentration of 100 μg/ml in 5% ethanol. These were individually added to lysis buffer or previously incubated with aliquots of the extract, as indicated.

Cytotoxic assays. Human peripheral blood mononuclear cells (PBMC) or peripheral blood granulocytes (PBG) were obtained by centrifugation of blood over a discontinuous Ficoll-Hypaque density gradient, as described by Bicalho et al. (9). The murine macrophage-like cell line J774 was also used. Cells were washed three times in PBS and resuspended in lysis buffer to 10^6 cells per ml. Aliquots (200 μl) of cell suspension were incubated with 10 μl of promastigote cExt serially diluted at 37°C for 30 min. Cells were counted in the presence of the vital dye Erythrosin B (Riedel de Haën), and membrane damage was evaluated by permeability to this dye. Alternatively, J774 cells, cultured in 96-well flat-bottomed microplates and incubated with promastigote cExt under the conditions described above, were assayed for membrane leakage by a lactate dehydrogenase release assay (Cytotox; Promega) according to the manufacturer's instructions.

All experiments in this study were carried out in duplicate, and the results shown are representative of three to five experiments.

RESULTS

Promastigotes have optimal hemolytic activity at pH 5.0 to 5.5 and 37°C. As we have previously reported, promastigotes of *L. amazonensis* IFLA/PA/67/PH8 are hemolytic when incubated with RBC at 37°C in acetate buffer (pH 5.5). Lysis was observed only after 5 h, and boiled parasites failed to lyse cells (29). As a matter of fact, the pH and temperature mentioned above are inadequate for promastigote survival, and now we show that lysis is detected as parasites die, even when a richer medium, such as RPMI 1640, is used (Table 1). Sodium azide (5 mM) and 2-deoxy-D-glucose (50 mM) failed to inhibit lysis; in fact, these metabolic inhibitors accelerated the death of parasites, increasing the levels of hemolysis (Table 1). Incubation at neutral pH and 25°C, conditions compatible with promastigote life, did not produce hemolysis, even after stimulation with the calcium ionophores A23187 (2 μg/ml) and ionomicin (2 μM) (Table 1).

We have also shown that cExts of freeze-thaw-disrupted parasites are much more rapid and efficient in causing lysis. Within only 5 min, RBC were lysed to the same extent as they were with 5 h of incubation with parasites, and within 20 min, all cells were lysed, as opposed to 80% hemolysis by parasites in 6 h (29). We show here that HuRBC, sheep RBC, and rabbit RBC are equally susceptible to lysis by cExt (Table 2), presenting comparable dose-response curves (not shown).

Hemolytic activity was also found in promastigotes of another strain of *L. amazonensis*, LTB0016 (not shown), and in another *Leishmania* species, *L. major* (WR309) (Fig. 1). Interestingly, however, two representative species of the subgenus *Viannia* presented little or no hemolytic activity. *L. panamensis*

TABLE 2. Lytic activities of promastigote extracts for RBC of different species and effects of different treatments

Target RBC	Type of extract[a]	Treatment	% Lysis ± SD
Sheep	cExt	None	92.3 ± 6.0
Rabbit	cExt	None	97.2 ± 2.3
Human	cExt	None	98.2 ± 1.5
Human	sExt	100°C, 5 min	10.1 ± 3.3
Human	sExt	4 mg of trypsin ml^{-1}, 37°C, 4 h[b]	26.4 ± 7.2
Human	sExt	4 mg of pronase ml^{-1}, 37°C, 4 h[b]	21.5 ± 4.6
Human	cExt	100 μg of Rosenthal ml^{-1}, 37°C, 1 h[b]	94.7 ± 3.2
Human	cExt	10 mM EDTA[c]	98.2 ± 1.6
Human	cExt	10 mM EGTA[c]	96.0 ± 3.9
Human	cExt	1–5 mM $CaCl_2$[c]	92.2 ± 3.7
Human	cExt	1–5 mM $MgCl_2$[c]	95.2 ± 3.5
Human	cExt	1–5 mM $ZnSO_4$[c]	95.5 ± 2.4
Human	cExt	1 mM PMSF	88.4 ± 6.4
Human	cExt	0.1 mM E-64[c]	98.0 ± 1.9
Human	cExt	5 mM *o*-phenanthroline[c]	98.3 ± 1.5

[a] At 1:40 final dilution.
[b] Previously incubated with cExt.
[c] Added to lysis buffer.

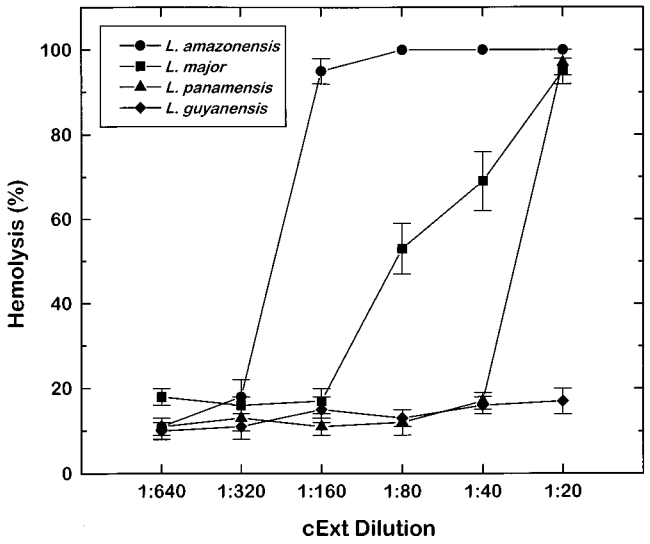

FIG. 1. Hemolytic activities of different species of *Leishmania*. HuRBC were incubated with serially diluted promastigote cExts of the indicated species at pH 5.5 for 30 min at 37°C. Spontaneous hemolysis was approximately 15%. Data are the means ± standard deviations (SD) of duplicates.

presented far less activity compared with that of *L. amazonensis* under the same conditions, whereas it was completely absent in *L. guyanensis* (Fig. 1) from any stage of promastigote growth (first to seventh day), at any pH (4.0 to 8.0), or at any temperature (0 to 37°C) (data not shown). Hence, all subsequent experiments were carried out with *L. amazonensis.*

To investigate the optimal pH for the hemolytic activity, we incubated HuRBC with *L. amazonensis* promastigote cExts at various pHs for 30 min at 37°C. The parasite lytic activity was maximal at pH 5.0 to 5.5, although lysis was still observed at up to pH 7.0 (Fig. 2A). At pH 4.5, hemolytic activity cannot be accurately evaluated because of extensive spontaneous hemolysis.

Comparing the hemolytic activities at different temperatures at pH 5.5, we observed that the lytic process is temperature dependent. At 0°C, lysis did not occur at all, and at 15°C, hemolytic activity was barely detectable; however, at 25°C, lysis was evident and increased with the rise in temperature (Fig. 2B), showing that it requires energy to occur. Similar results were obtained when assays were carried out at pH 7.0 (not shown).

The cytolytic factor is likely to be a soluble protein trapped in membrane vesicles. To investigate the solubility of the active molecule, we centrifuged the nucleus-free cExt of *L. amazonensis* promastigotes at 100,000 × *g* for 1 h and assayed the hemolytic activities of both supernatant and pellet. Figure 3 shows that all the hemolytic activity of cExt was retained in the insoluble membrane-rich pellet, with no activity observed in the soluble fraction (Fig. 3A), showing that the active molecule is associated with the parasite membrane. Solubilization of the parasite cytolysin could be achieved by treating the membrane-rich pellet with 0.6% CHAPS, a nonhemolytic concentration under the conditions of the assay. All of the activity was then found in the supernatant of the centrifuged (100,000 × *g*), detergent-treated material (sExt), whereas the remaining pellet was inactive (data not shown). To dissect the membrane compartment holding the cytolytic activity, we used the method of sodium carbonate (18) or EDTA (39) extraction, with subsequent centrifugation at 100,000 × *g* for 1 h. Previous reports have shown that the treatment of membrane fractions with

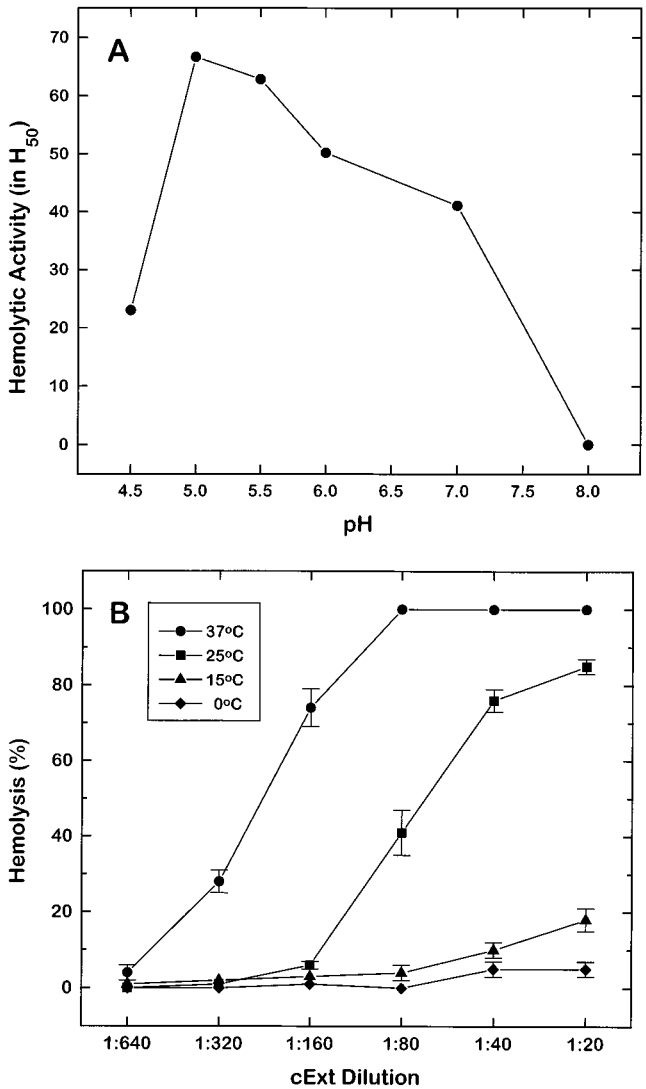

FIG. 2. Optimal pH and temperature for the hemolytic activity of *L. amazonensis*. (A) HuRBC were incubated with serially diluted promastigote cExts at the indicated pHs for 30 min at 37°C. For each pH, spontaneous hemolysis was subtracted from cExt-mediated hemolysis and activity has been reported as H_{50} (see Materials and Methods). (B) HuRBC were incubated with serially diluted promastigote cExts at pH 5.5 for 30 min at 0, 15, 25, and 37°C. Spontaneous hemolysis was approximately 5%. Data are the means ± SD of duplicates.

sodium carbonate (100 mM, pH 11.5) opens membrane vesicles, releasing soluble lumenal proteins, and removes peripheral membrane proteins, while leaving integral membrane proteins within the particulate fraction (18, 21, 22, 51). On the other hand, EDTA (5 mM) is known to remove peripheral membrane proteins, possibly without unsealing closed vesicles (18, 39). The treatment of the promastigote membrane-rich insoluble fraction of cExt (Fig. 3A, gray bar) with 100 mM sodium carbonate released nearly all the hemolytic activity to the supernatant (Fig. 3B). Conversely, the lytic molecule remained mostly in the insoluble fraction after the treatment of membranes with 5 mM EDTA (Fig. 3C). These results indicate that the cytolysin is neither an integral nor a peripheral membrane molecule; it is probably soluble and localized inside membranous vesicles. That this molecule is a protein was demonstrated by the fact that sExt boiled for 5 min or incubated

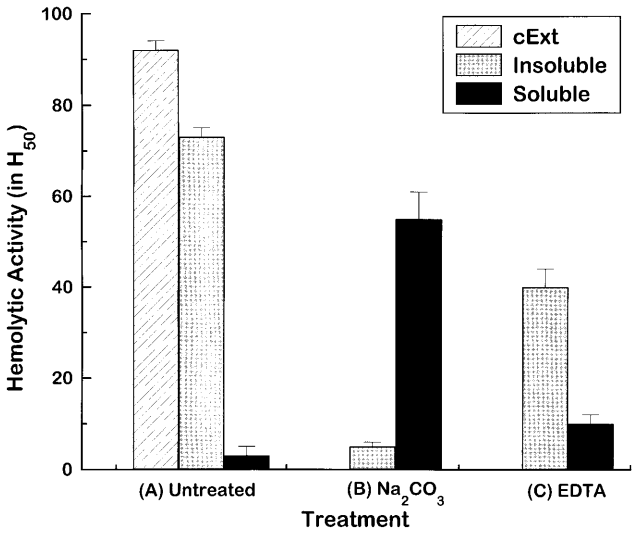

FIG. 3. Solubility of the hemolytic molecule of *L. amazonensis*. (A) Promastigote cExt was spun at 100,000 × *g* for 1 h. Hemolytic activity at pH 5.5 was assayed in both the supernatant (soluble) and pellet (insoluble), with the latter resuspended to the original volume with 10 mM Tris-HCl, pH 7.5. The insoluble fraction of cExt was treated with 100 mM of Na_2CO_3 for 30 min at 0°C (B) or 5 mM EDTA for 1 h at 25°C (C). After centrifugation at 100,000 × *g* for 1 h, the hemolytic activity was determined in both soluble and insoluble fractions. Hemolytic activity has been reported as H_{50} (see Material and Methods). Hemolysis in mock-treated, pH-adjusted controls was approximately 5, 16, and 15% in panels A, B, and C, respectively. Data are the means ± SD of duplicates.

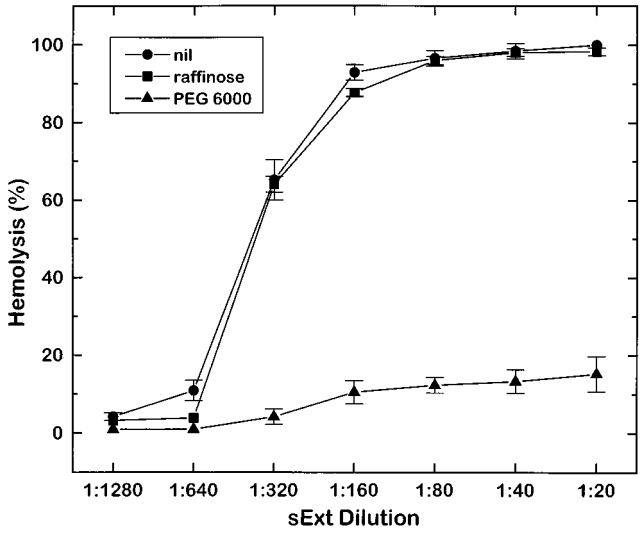

FIG. 4. Osmotic protection in hemolysis mediated by *L. amazonensis*. HuRBC were incubated with serially diluted promastigote sExt at pH 5.5 for 30 min at 37°C with or without (nil) 30 mM raffinose or PEG 6000. Spontaneous hemolysis was approximately 10%. Data are the means ± SD of duplicates.

with either trypsin or pronase completely lost its hemolytic activity (Table 2).

Hemolysis is colloid osmotic. In an attempt to identify the mechanism whereby the target cell membrane is damaged, we investigated the possibility that structural plasma membrane components could be degraded by phospholipases or proteases of this parasite. Neither inhibitors of phospholipase A_2, such as Rosenthal's reagent (100 μg) or EDTA or EGTA (10 mM), nor phospholipase activators, such as Ca^{2+}, Mg^{2+}, or Zn^{2+} (1 to 5 mM), altered the lytic potential of the parasite protein (Table 2). Likewise, the protease inhibitors PMSF and E-64 and the Zn^{2+} chelator *o*-phenanthroline did not affect the hemolytic activity of cExt (Table 2). These results indicate that the hemolytic protein has neither phospholipase A_2 nor protease behavior and that Ca^{2+}, Mg^{2+}, and Zn^{2+} are not required for lysis to occur.

The following experiment was then performed in order to examine the hypothesis that the cell damage caused by the *L. amazonensis* hemolytic protein is a result of pore formation on target cell membranes. PFPs lyse cells by creating transmembrane channels that allow ions and small molecules to pass freely across the bilayer. The high concentration of macromolecules inside the cell generates osmotic pressure, causing a water influx that leads the cell to burst. This process, known as colloid-osmotic lysis, can be prevented by the addition of macromolecules to the extracellular compartment to compensate the osmotic imbalance (55). We carried out a hemolytic assay with sExt in the presence of 30 mM raffinose or PEG (molecular mass of 6,000 g/mol). As shown in Fig. 4, PEG completely inhibited hemolysis mediated by sExt, whereas raffinose, a molecule with a molecular mass of 504 g/mol, did not protect RBC from lysis. This result suggests the colloid-osmotic nature of lysis, which is indicative of pore formation on the target cell membranes. The inhibition observed was not due to inactivation of the lytic protein by PEG, because lysis was restored after RBC were washed and resuspended in lysis buffer without PEG (data not shown). This result also suggests that the putative PFP binds to the target cell membrane before causing lysis.

The expression of the putative pore-forming activity seems to be regulated during promastigote growth in vitro. During the transition from the logarithmic stage to the stationary stage of growth in vitro, *Leishmania* promastigotes undergo a differentiation from complement-sensitive, noninfective parasites to complement-resistant, infective parasites (17, 28, 45, 46). This process is accompanied by the expression of various molecules (20, 23, 30, 35, 43, 44). To investigate whether the cytolytic activity is expressed differently throughout parasite development, we monitored the hemolytic activities of promastigotes during one cycle of growth in vitro. The hemolytic activity of cExt was low at the lag phase but gradually increased during the logarithmic phase. A peak was reached by the fourth or fifth day of growth, the beginning of the stationary phase, and the lytic activity returned to low levels as parasites grew older in the late stationary phase (Fig. 5). These results show that the expression of cytolytic activity seems to be regulated during parasite development.

The lytic factor is also present in amastigotes and is cytotoxic for macrophages. Both pH and temperature findings indicated that the lytic molecule could act at different stages of the parasite life cycle. However, the best conditions to reach maximum activity coincide with the temperature and pH found inside the mammalian host (6). Therefore, we examined whether the intracellular stage of this parasite also has the cytolytic factor and whether mammalian nucleated cells, particularly macrophages, are susceptible to lysis mediated by this molecule. Using parasites from lesions of infected mice, we found that amastigotes (not shown) and amastigote cExt (Fig. 6A) presented hemolytic activities comparable to that of promastigotes. Promastigote cExt was cytotoxic for human PBMC and PBG, as well as for the mouse macrophage-like cell line J774, at pH 5.5. As shown in Fig. 6B, cell membrane damage, as evaluated by permeability to the vital dye Erythrosin B (PBMC, PBG, and macrophages) or by the release of lactate dehydrogenase (macrophages), was evident. Taken together,

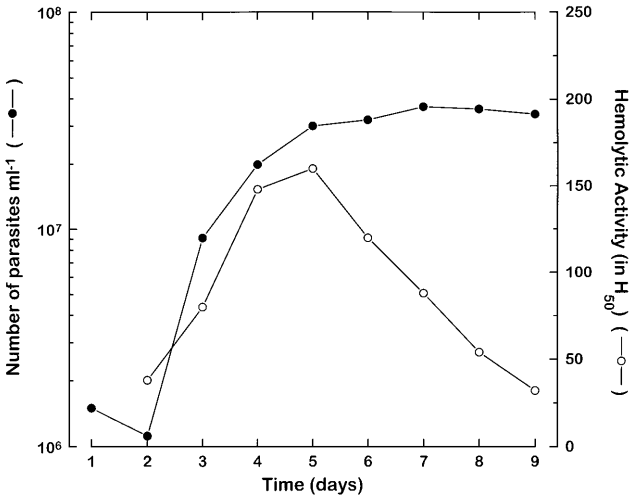

FIG. 5. Expression of the lytic activity during *L. amazonensis* promastigote growth in vitro. HuRBC were incubated with serially diluted sExt, obtained from the same number of parasites, on each day of culture at pH 5.5 for 30 min at 37°C. Hemolytic activity has been reported as H_{50} (see Materials and Methods). Spontaneous hemolysis was approximately 5%.

these results corroborate the notion that the cytolytic factor may have a functional role inside the vertebrate host.

DISCUSSION

We have described here a cytolysin of *Leishmania* spp. optimally active at pH 5.0 to 5.5 and at 37°C. We first detected this lytic activity in *L. amazonensis* by using the promastigote stage of this parasite and sheep RBC as targets. We showed that freeze-thaw extracts of this parasite were hemolytic almost immediately. On the other hand, nondisrupted parasites required 5 to 6 h to lyse RBC, whereas boiled parasites failed to lyse cells (29). We now have shown that the *L. amazonensis* lysin is a protein also found in amastigotes and also active against nucleated cells. Furthermore, we report here that in promastigotes, it seems to be localized inside membranous vesicles, but this stage of the parasite does not seem to secrete the molecule. We have presented indicative evidence that cytolysis is due to pore formation in the target membrane.

The hemolytic activity of promastigotes has also been found in *Leishmania* species other than *L. amazonensis*. We have shown that *L. major* presents considerable hemolytic activity at acid pH, although not as high as that in *L. amazonensis* (Fig. 1). O'Daly and Aso (31), using neutral pH, detected hemolytic activities in *L. mexicana* and *L. donovani*. However, no characterization of these hemolysins was reported, except that they were contained in the insoluble fraction of parasite extracts and that, in *L. mexicana*, the activity was associated with both lipid and protein phases of the insoluble fraction. Chakravarty et al. (12) showed that *L. donovani* promastigotes, autolysed overnight at pH 5.8 and 25°C, were lytic to HuRBC and rabbit RBC in a temperature-dependent manner but, as opposed to our results, did not affect sheep RBC.

One interesting observation is that the presence of cytolytic activity does not seem to be a feature shared by all species of the genus *Leishmania*. While these activities were found in members of the subgenus *Leishmania*, two members of the subgenus *Viannia* behaved otherwise; *L. panamensis* was poorly active, and *L. guyanensis* was inactive in causing cell lysis

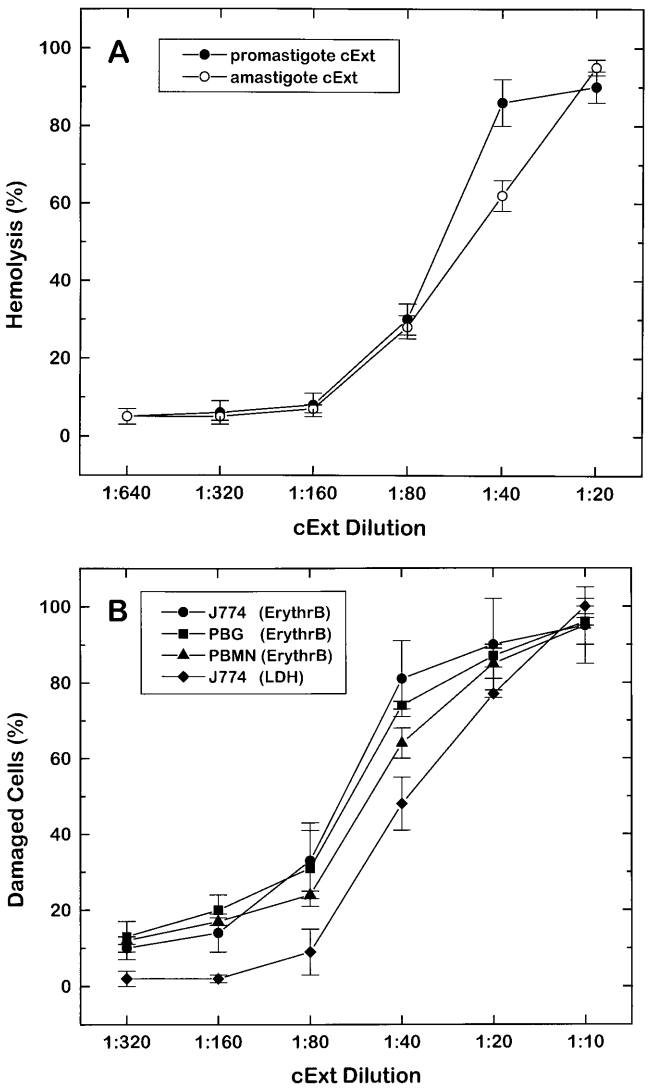

FIG. 6. Presence of the putative PFP in *L. amazonensis* amastigotes and lytic activity against nucleated cells. HuRBC (A) or PBMC, PBG, and cells of the murine macrophage-like cell line J774 (B) were incubated with serially diluted amastigote (A) or promastigote (A and B) cExts at pH 5.5 for 30 min at 37°C. Membrane damage of PBMC, PBG, and J774 cells was evaluated by permeability to Erythrosin B (ErythrB) or lactate dehydrogenase (LDH) leakage, as indicated. Spontaneous hemolysis was approximately 5%. Damaged cells in the absence of cExt did not exceed 12%. Data are the means ± SD of duplicates.

(Fig. 1). Systematic comparative studies are currently under way to verify the consistency of this dichotomy.

The abrogation of the hemolytic activities of *L. amazonensis* promastigote extracts by boiling or by treatment with trypsin or pronase (Table 2) demonstrated that the lytic molecule is a protein. Heat lability and protease sensitivity are also characteristics of the *L. donovani* hemolysin (12). The *L. amazonensis* cytolytic protein, like *L. donovani* (12, 31) and *L. mexicana* (31) hemolysins, was initially localized to the insoluble membrane-rich fraction (Fig. 3A). Here, however, we have shown that it was readily solubilized by treatment with sodium carbonate, which removes peripheral membrane proteins and unseals vesicle membranes (Fig. 3B). On the other hand, it remained mostly insoluble after EDTA treatment, which is thought to remove peripheral membrane proteins only (Fig. 3C). Therefore, the *L. amazonensis* promastigote cytolysin is likely to be

a soluble protein confined in membranous structures, e.g., lysosomes or other cytoplasmic vesicles such as megasomes (38, 52). Because the solubility of this cytolysin is preserved in detergent concentrations below the critical micelle concentration, we can indeed deduce that in experiments in which we used sExt diluted more than 20-fold, the molecule was already soluble but trapped inside vesicles.

From our previous experiments (29), we could not interpret how lysis was produced by *L. amazonensis* promastigotes. Although the absence of hemolytic activity in heat-killed promastigotes suggested an active role for the parasites (29), the delayed kinetics (5 to 6 h) do not fit well with the hypothesis of secretion of the cytolysin by promastigotes. This hypothesis is also refuted by our present findings that the metabolic inhibitors sodium azide and 2-deoxy-D-glucose failed to inhibit lysis (Table 1). Furthermore, cell-free supernatants of parasites incubated without RBC do not present hemolytic activities (36), indicating that promastigotes do not spontaneously secrete or release the active molecule in a soluble form. We now know that the appearance of hemolytic activity coincides with the death of the parasites (Table 1). In fact, pH 5.5 and 37°C are the conditions appropriate for amastigotes, but not for promastigotes. We know that rupturing the parasites exposed the hemolytic activity (29). Similarly, it is likely that the adverse conditions disturb the integrity of the plasma membrane, making available the cytolysin-containing vesicles. Under conditions compatible with promastigote survival (pH 7.0, 25°C), however, lysis was not observed, even when parasites were stimulated with the calcium ionophores A23187 and ionomicin (Table 1). We are currently investigating whether amastigotes can secrete this hemolysin at pH 5.5 and 37°C.

Leishmania spp. are parasites notably rich in proteases (11, 52). The most abundant promastigote surface protease is the metalloprotease gp63, which is active at acid pH and found in all *Leishmania* species (11). Lipolytic enzymes, such as phospholipases, are also present in certain species of *Leishmania* (16). Therefore, it was conceivable that proteolysis or phospholipid breakdown could account for the hemolysis mediated by *L. amazonensis*. However, the treatment of parasite extracts with protease inhibitors did not impair their lytic activities (Table 2), suggesting that no serine protease (inhibited by PMSF), cysteine protease (inhibited by E-64), or metalloprotease (inhibited by *o*-phenanthroline, EDTA, or EGTA) is involved in the lytic process. One can particularly rule out the participation of gp63 since *o*-phenanthroline, its specific inhibitor, did not reduce the parasite lytic activity. Moreover, phospholipase A_2 inhibitors and activators did not affect the hemolytic activities of parasite extracts either (Table 2), suggesting that no phospholipase A_2-like activity is involved in this cytolytic process. Detergent-like activity has already been described for some lytic proteins, such as the bee venom melittin (48), *Escherichia coli* alpha-hemolysin (34), and a toxin from a sea anemone (49). This mechanism was also ruled out for the *L. amazonensis* lytic activity. Whereas detergents are usually effective instantly at low temperatures, parasite extracts were totally inactive at 0°C for 30 min (Fig. 1B) and up to more than 1 h (data not shown). These results show that *L. amazonensis*-induced hemolysis does not seem to be a consequence of degradation of structural components of the target membrane by phospholipases, proteases, or detergent-like molecules.

The osmotic protection against sExt-mediated hemolysis induced by PEG (6,000 g/mol) but not by raffinose (504 g/mol) (Fig. 4) indicated that the lysis is colloid osmotic because of the generation of channels in the target cell membrane (33, 55). This suggests that the *L. amazonensis* lytic protein is a pore-forming molecule. Preliminary experiments using the patch-clamp technique have provided additional information that supports the pore formation mechanism (27). The inhibition of cell lysis by PEG 6000 implies its nontransferability from the extracellular compartment to the intracellular compartment, and the macromolecule's effective diameter is taken to exceed that of the functional pore. Thus, one can also infer that the membrane lesions caused by sExt in 30 min are smaller than 6.1 nm, the average diameter of PEG 6000 according to its Einstein-Stokes hydrodynamic radius (47). Lysis is Ca^{2+}, Mg^{2+}, and Zn^{2+} independent (Table 2) but requires energy to proceed (Fig. 2B), and the parasite putative PFP seems to bind to the cell membrane before causing lysis (see Results). This occurs in an unrestricted fashion, since both RBC and nucleated cells from different species were equally susceptible to lysis (Fig. 6B; Table 2).

There has been increasing evidence that parasites use PFPs to interact with and/or damage their hosts (4, 14). Among them are the bacteria *E. coli* (7, 40), members of the genera *Bacillus*, *Clostridium*, and *Streptococcus* (50), *Staphylococcus aureus* (8), *Actinobacillus actinomycetemcomitans* (54), and *Listeria monocytogenes* (10, 37) and the protozoans *Entamoeba hystolytica* (24, 57), *Naegleria fowleri* (19, 56), *Trypanosoma cruzi* (2), and *Plasmodium falciparum* (15). It is currently thought that these proteins play important roles in pathogenesis either by directly damaging the host cell membrane or by facilitating evasion from the immune system (3, 32).

Leishmania spp. are adapted to cope with the following two different environments: the alimentary tract of the invertebrate host, where the pH is probably above 7.0 and the temperature is 22 to 28°C, and the parasitophorous vacuole of vertebrate host macrophages, a functional acidic lysosomal compartment, with temperatures of 31 to 35°C in skin lesions and up to 37°C in visceral organs (58). The highest activity of the *L. amazonensis* putative PFP was achieved at pH 5.0 to 5.5 (Fig. 2A), a feature shared by PFPs from other intracellular organisms, such as listeriolysin O from *Listeria monocytogenes* and TC-TOX from *T. cruzi* (3). This suggests that the *L. amazonensis* putative PFP might fully express its activity inside the parasitophorous vacuole, where the pH varies from 4.7 to 5.2 (6). The fact that the lytic activities of parasite extracts were more prominent at 37°C than they were at lower temperatures (Fig. 2B) corroborates this notion. Another supporting finding is that the *L. amazonensis* cytolysin seems to be developmentally regulated, being maximally detected at the early stationary stage of in vitro-grown promastigotes (Fig. 5), just as they undergo differentiation into more-competent, vertebrate-infective forms (45, 46). In *L. donovani*, the hemolytic activity seems to be more effective in log-phase parasites than in stationary-phase parasites (12). Because no kinetics experiments were performed during the entire period of *L. donovani* development, it is still possible that the peak of activity coincides with the early stationary phase.

The presence of a putative PFP optimally active at acid pH and 37°C in *L. amazonensis* raises the question of its functional role in vivo. Listeriolysin O (10) and TC-TOX (2) have been implicated in the escape from the phagolysosome to the cytoplasm. *Leishmania* organisms do not leave the phagolysosome, surviving and multiplying inside this compartment (1). However, at further stages of the parasite's life cycle, both macrophage vacuolar and plasma membranes are disrupted (26, 42), releasing amastigotes, which are infective for neighboring cells. The mechanism of cell rupture is largely unknown and supposed to be merely mechanical. However, the parasitophorous vacuole is a vesicle that can harbor many parasites and can swell (13) or fuse with other phagocytic vesicles (53), occupying most of the host cell volume (6, 13). Thus, a mechanical rup-

ture of the membrane seems implausible; it is tempting to speculate that amastigotes may use the cytolytic molecule to actively disrupt host cells. This assumption is supported by the demonstration that *L. amazonensis* amastigotes also expressed lytic activities (Fig. 6A) and that macrophages were susceptible to cExt-mediated membrane damage (Fig. 6B). One has to bear in mind, though, that the outer membranes of the macrophages used as target cells and the inner faces of lysosomal membranes, or even the inner leaflet of the plasma membrane, differ in composition. On the other hand, however, amastigotes are lodged in the macrophages which adhere to the inner surfaces of phagolysosomes, which in turn are distended to the boundaries of the cell (6, 13). This intimate contact could favor the interaction of this cytolysin with inner membranes of the host cell. Since this lysin seems to be a lumenal protein, it is also possible that parasite vesicles fuse with host cell inner membranes, delivering the cytolysin onto its target.

The *L. amazonensis* cytolytic protein could also be functional at other stages of parasite development, since hemolytic activity can be detected in promastigotes at higher pHs and at lower temperatures (Fig. 2). It could either have an active role in the penetration of the host cell by the parasite or affect the permeability of the host cell membrane, causing localized ion fluxes that could in turn facilitate phagocytosis. Another possibility is that promastigotes use the hemolysin to obtain nutrients from RBC inside the gut of the insect vector, as already suggested by others (12). Naturally, further studies need to be completed in order to establish the exact function of the *Leishmania* cytolysin and its potential role in the pathogenesis of leishmaniasis.

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