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Acid Phosphatase Activity of Virulent and Avirulent Clones of *Leishmania donovani* Promastigotes

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Virulent and avirulent clones of *Leishmania donovani* promastigotes were examined for their acid phosphatase activity. The acid phosphatase activity of whole-cell lysates of virulent clones was 1.5 to 2.0 times higher than that of avirulent clones. Pellet fractions (260,000 × *g*, 30 min) from sonicated promastigotes of a virulent clone and an avirulent clone contained 60 and 40% of the total enzyme activity, respectively. Membrane-bound acid phosphatase was extracted with Triton X-100 from the pellet. This membrane-bound phosphatase activity was 2.4-fold higher in virulent organisms than in avirulent organisms. The membrane acid phosphatase exhibited two distinct bands on polyacrylamide gels stained for enzyme activity. One diffuse, faster-migrating band showed identical electrophoretic mobility in both virulent and avirulent clones, although a higher enzymatic activity was observed with the extract from virulent cells. In contrast, a slower-migrating band was different between the two clones in the mobility. These results suggest that membrane-bound acid phosphatase was quantitatively and qualitatively different between virulent and avirulent promastigotes of *L. donovani*. In addition, virulent cells produced a relatively high level of acid phosphatase throughout the growth in culture.

Promastigotes of *Leishmania donovani* contain small amounts of acid hydrolases except for acid phosphatase (9). This acid phosphomonoesterase activity has been demonstrated not only in the cytoplasmic vacuoles and vesicles but also on the outer surface membrane of the parasites (10). Remaley et al. (27) purified three membrane acid phosphatases from *L. donovani* promastigotes. They demonstrated that the major species, L-(+)-tartrate-resistant phosphatase, suppressed host phagocytic cells by reducing their ability to produce toxic oxygen metabolites (28). This finding suggests a role of the major membrane acid phosphatase for intracellular survival of the parasites.

Recently, we isolated virulent promastigote lines from an infectivity-reduced strain of *L. donovani* by serial mouse passages of the parasites (14). We also reported that virulent lines exhibited a high level of acid phosphatase activity relative to that of avirulent lines (13). In the present study, we extend our previous observations by showing that virulent promastigote clones of *L. donovani* possess a high level of the membrane-bound acid phosphatase activity and that there is a qualitative difference in this enzyme between virulent and avirulent organisms. In addition, we report here that the virulent cells produce a relatively high amount of acid phosphatase in comparison with avirulent cells throughout the growth of these promastigotes in culture.

MATERIALS AND METHODS

Parasites. Virulent promastigote lines were isolated from the infectivity-reduced 2S strain of *L. donovani* (originally isolated from a patient in Sudan in 1962) by serial alternate passages of the parasites in mice and in medium (14). A virulent line, 2S-25M, was cloned by limiting dilution, and additional virulent clones were obtained (13). Avirulent clones were also isolated from the 2S strain (13). Promastigotes were cultured in a monophasic undefined liquid medium with 5% defibrinated and hemolyzed rabbit blood at 25°C (24) and passaged every 1 to 2 weeks.

Determination of infectivity of promastigotes. Female BALB/c mice of 8 weeks of age were inoculated intravenously with 5×10^7 organisms harvested from stationary-phase cultures. Two weeks after the inoculation the number of amastigotes was counted per 500 liver cell nuclei in Giemsa-stained liver stamp preparations, and the number of Leishman-Donovan units in the liver was determined by the method of Bradley and Kirkley (2).

Isolation of membrane fraction. Crude membrane fractions of promastigotes were isolated as described by Glew et al. (9). All procedures were carried out at 4°C. Promastigotes of a virulent clone, 2S-25M-C2, and an avirulent clone, 2S-C2, were harvested from the stationary phase after cultivation for 7 days. Cells were washed three times with phosphate-buffered saline (pH 7.4). A washed pellet containing 1.59×10^9 or 1.74×10^9 cells was suspended in 1.0 ml of distilled water and sonicated at 20 kc for 2 min. The crude homogenate was subjected to centrifugation at 260,000 × *g* for 30 min to obtain a water-soluble fraction and a pellet fraction. The latter was extracted for 3 h by gentle stirring in 0.3 ml of 10 mM sodium citrate buffer (pH 6.5) containing 0.2% (vol/vol) Triton X-100. The Triton X-100 extract was centrifuged at 200,000 × *g* for 30 min. The resultant supernatant was set aside, and the pellet was extracted with 0.3 ml of the buffer as described above for an additional 5 h. The two Triton X-100 supernatant fractions were pooled and stored at −70°C until use.

Assay of acid phosphatase activity. Acid phosphatase activity was determined by a modification of the method of Saito and Suter (33) with promastigotes lysed by freezing-thawing. Promastigotes were washed with phosphate-buffered saline and then stored at −70°C until use at a concentration of 2×10^7 cells per ml of 0.1 M acetate buffer (pH 5.2) containing 1 mM $MgSO_4$. A typical assay mixture contained

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TABLE 1. Infectivity of clones of *L. donovani* promastigotes

Clone	Parasite burden in liver (LDU ± SD)[a]
2S-25M (uncloned)	2,270 ± 210
2S-25M-C1	2,980 ± 400
2S-25M-C2	2,740 ± 390
2S-25M-C5	2,520 ± 400
2S-25M-C8	1,980 ± 280
2S (uncloned)	8 ± 2
2S-C2	7 ± 6
2S-C3	8 ± 4
2S-C4	27 ± 20

[a] Four or five BALB/c mice were intravenously inoculated with 5×10^7 promastigotes, and the number of Leishman-Donovan units (LDU) in the liver was determined 2 weeks later.

cell lysate (equivalent to 10^6 cells), 0.05% Triton X-100, and 2 mM *p*-nitrophenyl phosphate in acetate buffer as described above. The reaction mixtures in 0.3 ml were incubated at 37°C for 30 to 40 min. The reactions were terminated by the addition of 0.8 ml of 1 N NaOH, and the release of *p*-nitrophenol (PNP) was estimated spectrophotometrically at 400 nm. For the crude membrane fractions, the standard assay was carried out for 20 min at 37°C in a 0.15-ml reaction mixture containing 5 mM *p*-nitrophenyl phosphate in 0.1 M acetate buffer (pH 5.0). Enzyme activity was expressed as nanomoles of PNP released per minute per milligram of protein or per 10^9 cells.

Protein concentration was measured by the method of Lowry et al. (22) or Peterson (26) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. Nondenaturing electrophoresis was performed on a 2-mm-thick gel apparatus with a 7% acrylamide separating gel and 3% acrylamide stacking gel at pH 8.3 in a system identical to that described by Laemmli (18), except that sodium dodecyl sulfate was omitted from gels and buffers. Water-soluble or Triton X-100 extract fractions were each mixed with an equal volume of 50 mM Tris hydrochloride buffer (pH 6.8) containing 15% sucrose before electrophoresis. After electrophoresis, the gels were rinsed in ice-cold 0.1 M acetate buffer (pH 4.0) for 15 min and then stained overnight at room temperature for enzyme activity in 0.1 M acetate buffer (pH 5.0) containing 1 mg of α-naphthyl acid phosphate per ml and 1 mg of Fast Garnet GBC salt (Sigma Chemical Co., St. Louis, Mo.) per ml (19).

RESULTS

Effect of Triton X-100 on acid phosphatase activity. To detect all available acid phosphatase activity, promastigotes lysed by freezing-thawing were used in the presence of various concentrations of Triton X-100. A maximum phosphatase activity was detected by the treatment of the cell lysate with 0.05% Triton X-100, which increased the activity up to 170% of that of the detergent-free control.

Acid phosphatase activity of promastigote clones. Virulent promastigote clones, which produced heavy parasite burden in the liver of mice (Table 1), and avirulent clones were assayed for their acid phosphatase activity. All of the four virulent clones derived from a virulent uncloned 2S-25M line exhibited a higher enzyme activity than did avirulent clones (Table 2). The mean specific activity of the lysed cells of virulent clones was 1.6 times higher than that of the avirulent clones. More than 80% (84 to 87%) of the phosphatase activity of virulent promastigotes remained in the presence of 0.5 mM sodium tartrate, whereas the tartrate-resistant enzyme of avirulent cells showed less than 75% (71 to 73%) of the total. These results indicate that the difference in acid phosphatase activity of whole-cell lysates between virulent and avirulent clones is mainly due to the difference in tartrate-resistant phosphatase, which is located on the surface membrane of promastigotes (27).

Acid phosphatase activity of membrane fractions. To further ascertain the difference in membrane acid phosphatase, crude membrane fractions were isolated and examined for the enzyme activity. Pellet fractions (260,000 × *g*, 30 min) from sonicated promastigotes of a virulent clone (2S-25M-C2) and an avirulent clone (2S-C2) contained 60 and 40% of the total acid phosphatase activity, respectively; approximately 20% of the total enzyme activity was lost during fractionation (Table 3). Membrane-bound acid phosphatase could be extracted with 0.2% Triton X-100 from the pellet fractions. The Triton X-100 supernatant fraction (200,000 × *g*, 30 min) of the 2S-25M-C2 cells exhibited a specific activity 2.4 times higher than that of 2S-C2 cells. These membrane phosphatases of the 2S-25M-C2 and 2S-C2 were both resistant to tartrate, showing 90 and 85% resistance in the presence of 0.5 mM sodium tartrate, respectively.

Acid phosphatase activity during growth in culture. The kinetics of acid phosphatase activity during the growth of promastigotes in culture was examined. The cells reached the midlogarithmic and stationary phases of growth at days 3 and 7, respectively (Fig. 1A). The acid phosphatase activity of organisms in the log phase was higher than that in the stationary phase; this was found for both virulent 2S-25M-C2 and avirulent 2S-C2 clones (Fig. 1B). The tartrate resistance of the enzyme activity reached a maximum on day 5 with 2S-25M-C2 and on day 3 with 2S-C2. Although it is unknown why log-phase promastigotes exhibit higher acid phosphatase activity than stationary cells, no inhibitory substance was detected in the promastigote lysates, as shown by mixing cell lysates prepared on day 3 and those on day 7 (data not shown). However, it is noteworthy that virulent promastigotes produced a high amount of acid phosphatase relative to that produced by avirulent cells throughout the growth in culture (Fig. 1B).

Electrophoretic patterns of acid phosphatase. To characterize the electrophoretic behavior of membrane-bound acid

TABLE 2. Acid phosphatase activity of virulent and avirulent clones of *L. donovani* promastigotes

Clone	Acid phosphatase activity[a]		
	Sp act[b]	% Tartrate resistant[c]	Activity per cell[d]
2S-25M (uncloned)	115.8 ± 3.8	84.1 ± 2.3	989 ± 48
2S-25M-C1	132.0 ± 24.0	85.8 ± 0.1	1,049 ± 267
2S-25M-C2	124.2 ± 8.3	86.8 ± 1.6	1,019 ± 35
2S-25M-C5	117.2 ± 15.7	84.4 ± 0.3	1,042 ± 23
2S-25M-C8	126.3 ± 10.3	84.8 ± 0.7	1,413 ± 306
2S (uncloned)	67.2 ± 2.0	71.0 ± 2.7	475 ± 73
2S-C2	82.1 ± 11.5	73.0 ± 2.8	615 ± 122
2S-C3	83.1 ± 13.9	72.9 ± 0.1	676 ± 124
2S-C4	62.9 ± 4.2	73.1 ± 1.6	645 ± 48

[a] Results are the means ± standard deviations of two experiments.
[b] Nanomoles of PNP liberated per minute per milligram of protein.
[c] Enzyme activity was measured in the presence of 0.5 mM sodium tartrate.
[d] Nanomoles of PNP liberated per minute per 10^9 cells.

TABLE 3. Acid phosphatase activity of crude fractions from virulent and avirulent clones of *L. donovani* promastigotes

Fraction	Protein (pg/cell)		Sp act[a]		Activity per cell[b]	
	2S-25M-C2	2S-C2	2S-25M-C2	2S-C2	2S-25M-C2	2S-C2
Crude homogenate	6.02	5.40	142.8	70.1	860	379
Water soluble	2.32	2.25	88.8	61.7	206	139
Pellet	3.57	2.78	138.7	54.0	495	150
Triton X-100 extract	1.59	1.40	202.9	85.9	323	120

[a] Nanomoles of PNP liberated per minute per milligram of protein.
[b] Nanomoles of PNP liberated per minute per 10^9 cells.

phosphatase of virulent and avirulent promastigotes, nondenaturing electrophoresis was performed with α-naphthyl acid phosphate as the substrate. A quantitative difference in the membrane phosphatase activity on the gels was observed between virulent 2S-25M-C2 and avirulent 2S-C2 clones; two- to fourfold higher activity was detected with Triton X-100 extract from virulent cells (Fig. 2, lanes A and B). The membrane phosphatase showed two distinct activity bands. One diffuse faster-migrating band made a ladder of multiple bands, and this was common to both virulent and avirulent clones. In contrast, the other slower-migrating band was different between the two clones in the electrophoretic mobility. The uppermost band of virulent cells migrated more anodally than did that of avirulent cells (Fig. 2, lanes A and B). These differences in electrophoretic mobility of the uppermost bands suggest a qualitative difference of the enzyme between virulent and avirulent cells, since it has been reported that significant electrophoretic differences of acid phosphatase on polyacrylamide gels were observed among strains and species of *Leishmania* (21). When water-soluble fractions were used, a quantitative difference between the two clones was not apparent (Fig. 2, lanes C and D). The banding pattern was, however, similar to that of membrane phosphatase. One diffuse faster-migrating band appeared to be common to both clones, and the other slower-migrating band showed different electrophoretic mobility between them.

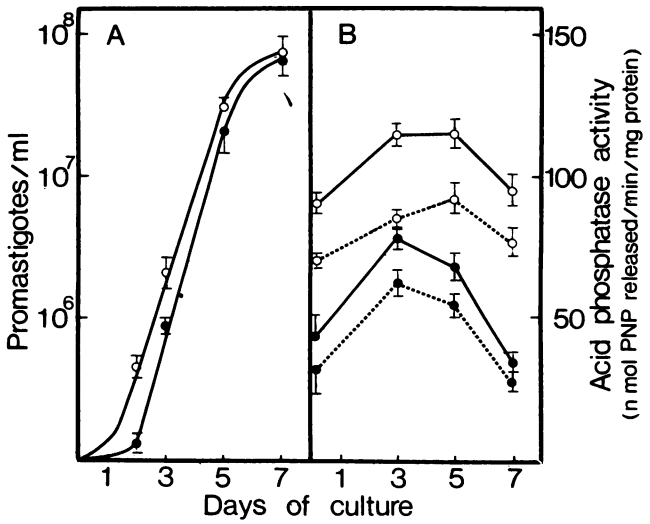

FIG. 1. Acid phosphatase activity during the growth of *L. donovani* promastigotes. Promastigotes of 2S-25M-C2 (○) and 2S-C2 (●) were grown in the Mansour liquid medium at 25°C. The number of motile promastigotes was counted with a hemacytometer (A). Promastigotes harvested at each culture day were lysed by freezing-thawing and by treatment with 0.05% Triton X-100. Lysed cells were then assayed for their acid phosphatase activity by using *p*-nitrophenyl phosphate as the substrate (B) in the absence and presence of 0.5 mM sodium tartrate, from which total (——) and tartrate-resistant (---) acid phosphatase activity was determined. Each point represents the mean ± standard error of three or four experiments.

DISCUSSION

In the present study, we report that virulent cloned promastigotes of *L. donovani* possess a relatively high level of membrane-bound, tartrate-resistant acid phosphatase activity as compared with cloned avirulent organisms. This result strongly suggests that membrane acid phosphatase is one of the factors relevant to the infectivity of *L. donovani* promastigotes.

Recently, many lines of evidence have suggested a pathophysiological role of tartrate-resistant acid phosphatase of *L. donovani*. A substantial amount of this enzyme is located on the external surface of promastigotes (10). This organism is known to contain only small amounts of acid hydrolases except for acid phosphatase (9). The cell surface phosphatase has a rather broad substrate specificity (9, 27) and is capable of catalyzing the dephosphorylation of phosphoproteins such as liver pyruvate kinase (27). Preincubation of human neutrophils and macrophages with the purified tar-

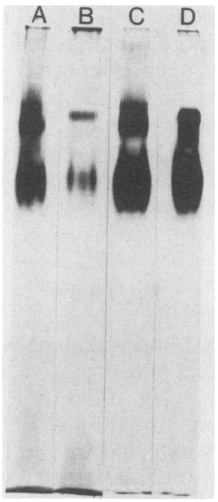

FIG. 2. Nondenaturing polyacrylamide gel electrophoresis of endogenous acid phosphatase of *L. donovani*. Approximately 100 μg of Triton X-100 extract from virulent clone 2S-25M-C2 (A) or avirulent clone 2S-C2 (B) or 100 μg of water-soluble fraction from 2S-25M-C2 (C) or 2S-C2 (D) was applied onto the gels. Electrophoresis was carried out on a 7% acrylamide gel stained with α-naphthyl acid phosphate and Fast Garnet GBC salt at pH 5.0 as described in Materials and Methods.

trate-resistant acid phosphatase markedly reduced the ability of these cells to produce toxic oxidative metabolites in response to stimulation with the activator formylated peptide (28). Furthermore, this leishmanial tartrate-resistant phosphatase is more resistant to denaturation of oxygen intermediates, superoxide anions, hydrogen peroxide, and hypochlorous acid in comparison with other acid phosphatases from plants and animal sources (32). These findings suggest that leishmanial membrane acid phosphatase may make dephosphorylation of phosphoproteins on the host phagocytic cell surface, thereby suppressing the production of oxygen metabolites by those cells and helping the survival of the parasites in the host. Our previous study showed that susceptibility to hydrogen peroxide was not different between virulent and avirulent promastigotes under cell-free conditions in the presence of lactoperoxidase and potassium iodide (13). The present results showed that virulent clones of *L. donovani* promastigotes exhibited twice as much tartrate-resistant acid phosphatase activity as did avirulent cells.

The surface membrane-bound acid phosphatase has been reported in *Leishmania* species (10, 12) and other trypanosomatids (12, 25). Three distinct phosphatases have been isolated from the plasma membrane of *L. donovani* promastigotes, and they showed different electrophoretic mobilities in the nondenaturing polyacrylamide gel electrophoresis (27). The major L-(+)-tartrate-resistant phosphatase (ACP-P_1) had the greatest anodal mobility at pH 8.8 (27). We observed two distinct electrophoretic bands when Triton X-100 extracts were electrophoresed under nondenaturing conditions and then stained with α-naphthyl acid phosphate and Fast Garnet GBC salt. One diffuse faster-migrating band was common to both virulent and avirulent clones, although the extract from virulent cells showed a higher enzyme activity. In contrast, the other slower-migrating band was different in electrophoretic mobility between the two clones; an extract from the virulent clone migrated more anodally than did that from the avirulent clone. It is unknown whether this species corresponds to either of two membrane phosphatases described by others (27) or results from the same enzyme heterogeneity as that reported for the extracellular acid phosphatase of *L. donovani* spp. (20, 21). Although the possibility cannot be excluded that the slower-migrating band may result from degradative changes of the enzyme by endogenous proteases, the present data suggest that membrane-bound acid phosphatase is both quantitatively and qualitatively different between virulent and avirulent promastigotes of *L. donovani*.

There have been comparative studies on virulent and avirulent strains or isolates of *Leishmania* at the molecular and biochemical levels. Observations of lectin-binding activity among virulent and avirulent strains of leishmanial promastigotes suggest that alteration of carbohydrate constituents of the surface glycoproteins or glycoproteins themselves is associated with infectivity of the parasites (4, 6). Virulent 2S-25M-C1 and 2S-25M-C2 clones showed marked agglutination with concanavalin A compared with the avirulent 2S-C2 and 2S-C3 clones (unpublished data). This finding is consistent with our observation of a quantitative difference in their surface membrane acid phosphatase, which is known to bind to concanavalin A (27). By two-dimentional gel electrophoresis of proteins and the fingerprinting of kinetoplast DNA, no significant difference was detected between infective and noninfective clones of *Leishmania tropica* promastigotes (11). Endogenous factors affecting leishmanial virulence have been independently investigated. Recently it has been reported that a major *Leishmania* surface membrane protein is an alkaline protease (1, 7) or an acid protease (3). This acid protease (gp63) is associated with the virulence of *Leishmania mexicana* subsp. *amazonensis*, suggesting that gp63 is a virulent factor of *Leishmania* spp. (3). In addition, the expression of gp63 is also associated with *N*-acetylglucosamine-1-phosphotransferase activity (16); thus, leishmanial virulence may be related to amplification or expression of gene(s) encoding enzymes involved in the regulation of *N*-glycosylation of parasite proteins (15). Since surface acid phosphatases are glycoproteins, the expression of the enzymes might be controlled by N glycosylation.

Another aspect of leishmanial infectivity has been established by evidence for development of noninfective-stage promastigotes to infective-stage cells during their growth in culture (5, 8, 17, 30) or in the sandfly vector (30, 31). An association between infective-stage promastigotes in the stationary phase and expression of an antigen on the surface (17, 29) or their enzymatic activities (23) has been reported. Since the membrane acid phosphatase was produced by both virulent 2S-25M-C2 and avirulent 2S-C2 clones to different degrees throughout the in vitro growth, acid phosphatase activity may be associated with strain-dependent infectivity rather than growth-cycle-dependent infectivity of *L. donovani* promastigotes.

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