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Localization and Activity of Various Lysosomal Proteases in *Leishmania amazonensis*-Infected Macrophages

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In mammalian hosts, *Leishmania* amastigotes are obligatory intracellular parasites of macrophages and multiply within parasitophorous vacuoles of phagolysosomal origin. To understand how they escape the harmful strategies developed by macrophages to kill ingested microorganisms, it is important to obtain information on the functional state of parasitophorous vacuole. For this purpose, we studied the intracellular distribution and activity of host lysosomal proteases in rat bone marrow-derived macrophages infected with *Leishmania amazonensis* amastigotes. Localization of cathepsins B, H, L, and D was investigated by using specific immunoglobulins. In uninfected macrophages, these enzymes were located in perinuclear granules (most of them were probably secondary lysosomes) which, after infection, disappeared progressively. In infected macrophages, cathepsins were detected mainly in the parasitophorous vacuoles, suggesting that the missing secondary lysosomes had fused with these organelles. Biochemical assays of various proteases (cathepsins B, H, and D and dipeptidyl peptidases I and II) showed that infection was accompanied by a progressive increase of all activities tested, except that of dipeptidyl peptidase II, which remained constant. No more than 1 to 10% of these activities could be attributed to amastigotes. These data indicate that (i) *Leishmania* infection is followed by an increased synthesis and/or a reduced catabolism of host lysosomal proteases, and (ii) amastigotes grow in a compartment rich in apparently fully active proteases. Unexpectedly, it was found that infected and uninfected macrophages degraded endocytosed proteins similarly. The lack of correlation in infected macrophages between increase of protease activities and catabolism of exogenous proteins could be linked to the huge increase in volume of the lysosomal compartment.

Leishmania amastigotes are intracellular parasites of mammals which multiply almost exclusively in macrophages within organelles of phagolysosomal origin, termed parasitophorous vacuoles (PV) (31).

How *Leishmania* amastigotes survive within the potentially harsh environment of the phagolysosomal compartment remains an intriguing and unresolved question. Several hypotheses have recently been proposed concerning mechanisms whereby *Leishmania* inhibit or withstand toxic processes generated in these organelles. Thus, for their phagocytosis they could use macrophage receptors not involved in the triggering of respiratory burst (29, 33). Impairment of the oxidative metabolism by intracellular *Leishmania* and detoxification of oxygen intermediates by isolated amastigotes have also been documented (11, 26, 29). Otherwise, some studies suggest that *Leishmania* amastigotes are well adapted to the phagolysosomal compartment and are resistant to at least some of its cytotoxic mechanisms. Indeed, PV were found to maintain an internal low pH (4a, 14; L. Rivas and K.-P. Chang, Abstr. MBL Gen. Meet., Biol. Bull. **165**:536, 1983). In addition, the presence in the lumen of PV of host cell-derived lysosomal acid phosphatase, trimetaphosphatase, and arylsulfatase has been demonstrated by cytochemistry (4). Determination of the same enzyme activities in macrophage lysates showed that they are either unaffected or increased after infection, suggesting that *Leishmania* cells do not reduce the amount or function of these enzymes (4). However, the fate of lysosomal proteases, which could be more harmful for the parasites, was not assessed in this previous study. Furthermore, nothing was known about the in situ activity of lysosomal enzymes after infection. To further our understanding of *Leishmania* survival within PV, we undertook the present study of *Leishmania amazonensis*-infected rat bone marrow-derived macrophages. We used immunocytochemistry to investigate the subcellular localization of four lysosomal cathepsins, namely, cathepsins B (EC 3.4.22.1), L (EC 3.4.22.15), H (EC 3.4.22.16), and D (EC 3.4.23.5), and we used quantitative assays to investigate the fate of five host cell lysosomal protease activities, namely, cathepsins B, H, and D, dipeptidyl peptidase I (DPP I; EC 3.4.14.1), and dipeptidyl peptidase II (DPP II; EC 3.4.14.2). We also investigated the in situ activity of lysosomal proteases, by measuring the ability of infected macrophages to degrade hen egg ovalbumin (OVA) and bovine serum albumin (BSA). The results indicate that after infection, lysosomal proteases are translocated in PV and that their activities, when measured in cell lysates, are either unaltered or greatly increased. Nevertheless, infected and uninfected macrophages express very similar proteolytic activities toward exogenously added proteins.

MATERIALS AND METHODS

Animals. Female BALB/c mice (2 to 4 months old) were purchased from Iffa Credo, St-Germain-sur-l'Arbresle, France, and male Fischer 344 rats (4 to 5 months old) were provided by the breeding center of the Pasteur Institute.

Parasites. *L. amazonensis* LV 79 (World Health Organization reference number MPRO/BR/72:M1841) was passaged in BALB/c mice. Amastigotes were isolated from lesions and purified as previously described (2).

Macrophage cultures. Rat bone marrow cells were cul-

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tured as described earlier (4) in Dulbecco modified Eagle minimum essential medium (Seromed, Berlin, Federal Republic of Germany) supplemented with 10% fetal calf serum (Boehringer GmbH, Mannheim, Federal Republic of Germany), 50 μg of gentamicin (Sigma Chemical Co., St. Louis, Mo.) per ml, and 10% L 929 fibroblast-conditioned medium (37) (culture medium). Five days later, adherent macrophages were harvested (4) and allowed to adhere to glass cover slips (diameter, 12 mm; 6×10^4 cells per cover slip) for light-microscopic studies or to tissue culture petri dishes (diameter, 35 mm; 0.5×10^6 to 1.5×10^6 cells per dish; Corning Glass Works, Corning, N.Y.) for biochemical studies. Cells were kept for 24 h at 37°C before *Leishmania* infection.

Infection of macrophage cultures. Macrophages were infected at a multiplicity of four parasites per host cell. Since *L. amazonensis* LV 79 is temperature sensitive (9), control and infected cultures were placed at 34°C in a 6% CO_2–94% air humidified atmosphere for different times (6, 24, 48, or 72 h). Macrophage and amastigote numbers present after various times of culture, as well as percentages of infected macrophages, were assessed as described previously (4).

Immunological reagents. Rabbit immunoglobulin G (IgG) directed against rat cathepsins B, L, H, and D were prepared as described earlier (40), and their specificities were tested by Western immunoblotting (12). Sheep anti-rabbit immunoglobulin and rabbit anti-OVA antibodies were purified by affinity chromatography. Sheep anti-rabbit immunoglobulin antibodies were labeled with β-galactosidase from *Escherichia coli* (a gift of A. Ullman, Institut Pasteur, Paris, France) or with horseradish peroxidase (grade I; Boehringer) by using a one-step and a two-step glutaraldehyde-coupling procedure, respectively (5, 6). Rhodamine-labeled sheep anti-rabbit immunoglobulin antibodies were purchased from Biosys, Compiègne, France.

Light-microscopic localization of lysosomal proteases. Macrophages were fixed with 4% paraformaldehyde (Merck-Schuchardt, Darmstadt, Federal Republic of Germany) in 0.1 M sodium cacodylate–HCl buffer (pH 7.4) for 1 h at room temperature. Cell preparations were then quenched with 50 mM NH_4 Cl in phosphate-buffered saline, permeabilized with phosphate-buffered saline containing 0.1 mg of saponin (Sigma) per ml and 10% normal rat serum, and sequentially incubated for 1 h at room temperature with anti-cathepsin IgG (70 to 200 μg/ml) (or anti-OVA antibodies for control preparations) and with rhodamine- or horseradish peroxidase-labeled conjugates (10 to 20 μg/ml). As additional controls, some cover slips were incubated only with conjugates. Phosphate-buffered saline containing 0.25% gelatin (Bio-Rad Laboratories, Richmond, Calif.) and 0.1 mg of saponin per ml was used as diluting medium for antibodies and conjugates. Cell-associated horseradish peroxidase activity was detected by using 3-amino-9-ethylcarbazole (Sigma) and H_2O_2 (Prolabo, Paris, France) as substrates (21).

Enzyme assays on cell lysates and culture supernatants. Macrophages or amastigotes were lysed in 5 mM sodium acetate-HCl (pH 5.5)–0.1% (vol/vol) Nonidet P-40 for titration of DPP II or in 50 mM Tris hydrochloride buffer (pH 7.5)–25 mM KCl–0.1% (vol/vol) Nonidet P-40 (TK-NP40) for titration of the other proteases. After 10 min at room temperature and 20 min at 4°C, lysates were spun at 10,000 × *g* for 30 min and supernatants were immediately stored at −80°C. To test the eventual release of protease activities by parasites, isolated amastigotes were kept at 34°C for various periods in RPMI 1640 medium containing 10% fetal calf serum and 50 μg of gentamicin per ml and buffered either at pH 5.3 with 25 mM 2-(*N*-morpholino)ethanesulfonic acid (Sigma) or at pH 7.3 with 25 mM 3-(*N*-morpholino)propanesulfonic acid (Sigma), after which cell supernatants and amastigotes were separated by centrifugation. Amastigotes were lysed as above, and cell lysates and supernatants were stored at −80°C until use.

Substrates used for cathepsin B, cathepsin H, DPP I, and DPP II assays were purchased from Bachem, Buddendorf, Switzerland, and were, respectively, *N*-α-carboxybenzoyl-L-arginyl-L-arginine-4-methoxy-2-naphthylamide tetraacetate (Z-Arg-Arg-MNA), L-arginine-4-methoxy-2-naphthylamide tosylate (arg-MNA), L-glycyl-L-arginine-4-methoxy-2-naphthylamide dihydrochloride (Gly-Arg-MNA), and L-lysyl-L-alanine-4-methoxy-2-naphthylamide dihydrobromide (Lys-Ala-MNA). They were dissolved in dimethylformamide (Sigma) and used in enzymatic assays at a final concentration of 1 mM (20, 34, 35). Bovine hemoglobin (Sigma) was used at a final concentration of 1% (wt/vol) as the substrate for measuring cathepsin D activity (8). Protocols used for cathepsin B, cathepsin H, DPP I, and DPP II assays were adapted from the method described by Smith et al. (36). Cathepsin B and DPP I were assayed in 100 mM potassium phosphate–1 mM EDTA buffer (pH 6.0 and 5.5, respectively) (34). Cathepsin H activity was assayed in 37.5 mM potassium phosphate–1 mM EDTA buffer (pH 6.8) (35). For the measurement of these three thiol-protease activities, 2 mM 2-mercaptoethylamide was added to the buffers as a reducing agent. DPP II activity was measured in 5 mM sodium acetate–HCl buffer (pH 5.5) (20). HCl (0.1 N) was added to stop the reactions. MNA released was detected by addition of 0.5 mg of tetrazotized *o*-dianisidine (Fast Blue B Salt; Sigma) per ml, and the A_{530} of the reaction product was measured. Enzyme activities are expressed as nanomoles or micromoles of MNA released per hour at 37°C and per milligram of protein or per 10^6 cells by using a reference curve constructed with known concentrations of free MNA (Bachem).

The cathepsin D assay was performed in 1 M sodium formate buffer (pH 3.1) (8). The reaction was terminated by the addition of trichloroacetic acid (3.5% wt/vol). Samples were then centrifuged, and each supernatant was filtered before the optical density at 280 nm was read. Results are expressed as arbitrary units, 1 unit being defined as the difference of 1.0 optical density unit at 280 nm between test and control filtrates. For all experiments, several kinds of control were used. They included reaction mixtures lacking substrate, lysate, or supernatant and complete reaction mixtures containing one of the following inhibitors: 1 μM leupeptin (Sigma), 5 mM iodoacetamide (Fluka, Buchs, Switzerland), 8 mM phenylmethylsulfonyl fluoride (Sigma), or 5 μM pepstatin (Serva, Heidelberg, Federal Republic of Germany).

Catabolism of endocytosed proteins. BSA (Grade V; Sigma) was radioiodinated by the Iodo-Gen method as specified by the manufacturer (Pierce Chemical Co., Rockford, Ill.). Macrophages infected for 48 h were incubated with labeled BSA (250 μg/ml; specific activity, 850 cpm/ng) for 2 h at 34°C, washed, and chased for 1 h at 34°C in fresh culture medium. Samples of cells and media were withdrawn before and after the chase period. Cells were lysed in TK-NP40, and 10% trichloroacetic acid-soluble radioactivity present in cell lysates and media was measured and expressed as a percentage of the total radioactivity associated with unchased cells.

Measurement of OVA (grade III; Sigma) degradation was performed by using an indirect enzyme immunometric assay

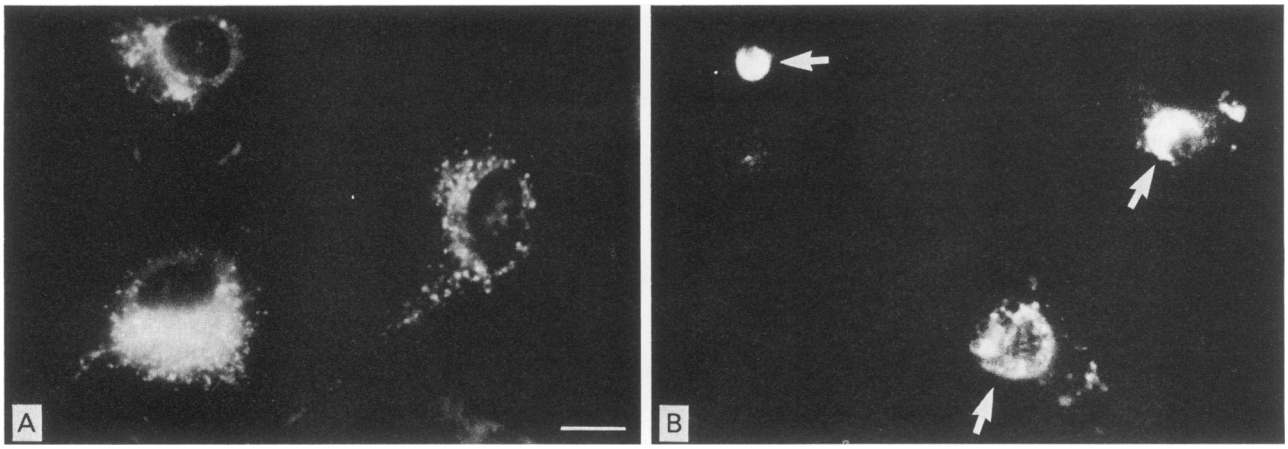

FIG. 1. Immunocytochemical detection of cathepsins in uninfected and infected macrophages. Cells, taken after 24 h of culture at 34°C, were incubated with rabbit anti-cathepsin H (A) or rabbit anti-cathepsin D (B) followed by rhodamine-labeled sheep anti-rabbit immunoglobulin antibodies. In uninfected cells cathepsins are located within perinuclear vesicles (panel A), whereas in infected macrophages they are detected mainly in the PV (panel B, arrows). Bar, 10 μm.

detailed elsewhere (3). Macrophages infected for 48 h were incubated for 3 h at 34°C with 5 mg of OVA per ml, washed, and chased for various times in fresh medium. Serial dilutions of media or cell lysates, prepared as described above, were mixed with rabbit anti-OVA antibodies. After being kept for 1 h at 37°C and overnight at 4°C, mixtures were added into wells of microdilution plates previously coated with OVA. After a 3-h incubation at 4°C followed by washing, β-galactosidase-linked sheep anti-rabbit immunoglobulin antibody and enzyme substrate (22) were sequentially added to the wells. Optical densities obtained were converted into amounts of OVA by reference to a standard curve made with known concentrations of OVA. Results were expressed as the percentage of the total amount of OVA associated with unchased cells.

Protein assay. Protein contents of amastigote or macrophage lysates were estimated by using the BCA reagent (Pierce). Bovine globulins (fraction II; Miles Laboratories, Inc., Kankakee, Ill.) were used as standards.

Amastigote viability assay. The viability of amastigotes was tested by reduction of 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) (28).

RESULTS

The degree of infection of macrophage cultures was assessed at various times after addition of parasites. The proportion of infected cells reached about 65% at 6 h and 85% from 24 h. Furthermore, the number of parasites per macrophage increased with time and reached 6, 9, and 15 after 6, 24, and 48 to 72 h of infection, respectively (data not shown). These results show that this in vitro model of infection is well suited for comparative biochemical assays of uninfected and infected macrophages.

Immunocytochemical localization of lysosomal proteases. The specificity of anti-cathepsin IgG used for cellular localization of proteases was tested by Western blot analysis of unreduced macrophage lysates. IgG directed to cathepsin D, B, H, and L recognized macrophage proteins of 42, 31, 29, and 31 kilodaltons, respectively, corresponding to mature lysosomal enzyme forms. These same IgGs did not stain Western blots of electrophoresed amastigote lysates (data not shown).

When used for immunocytochemistry, the four anticathepsin IgGs gave essentially similar results, whatever their fine specificity. In uninfected macrophages, cathepsins were detected in cytoplasmic granules that were located mainly in the perinuclear area (Fig. 1A) and that, for the most part, probably correspond to secondary lysosomes. After infection, the staining was considerably modified, since cathepsin-containing granules disappeared progressively and at the same time staining appeared in the PV. The appearance of the PV staining varied according to the size of the vacuoles. In relatively small PV, staining completely filled their lumen

TABLE 1. Specific activities of various proteases in uninfected macrophage and amastigote lysates[a]

Enzyme activity	Sp act (μmol or arbitrary unit/10^6 cells per h)[b] in:		Sp act (μmol or arbitrary unit/mg of protein per h)[b] in:	
	Macrophages	Amastigotes	Macrophages	Amastigotes
Cathepsin B	3.90 ± 0.27 (4)	0.013 ± 0.002 (4)	35.47 ± 2.85 (5)	6.24 ± 0.28 (3)
Cathepsin H	0.058 ± 0.005 (4)	0.0003 (1)	0.50 ± 0.05 (2)	0.14 (1)
DPP I	8.30 ± 1.96 (6)	0.013 ± 0.002 (7)	71.22 ± 13.86 (5)	6.57 ± 0.68 (3)
DPP II	2.14 ± 0.25 (4)	0 (2)	19.16 ± 0.84 (2)	0 (1)
Cathepsin D	0.33 ± 0.07 (3)	0.007 ± 0.001 (2)	3.09 ± 0.16 (3)	3.34 ± 0.82 (2)

[a] Uninfected macrophages derived from 5 -day bone marrow cultures and purified amastigotes were incubated at 34°C for 6 to 24 h before lysis.

[b] All protease activities are expressed in nanomoles or micromoles of reaction product (MNA) released per hour at 37°C and per 10^6 cells or per milligram of protein, except for cathepsin D, whose activity is expressed in arbitrary units. Amastigote lysate contained 2.08 ± 0.08 μg of protein per 10^6 cells (mean ± one standard deviation of three determinations), and macrophage lysates contained 114.39 ± 9.91 μg of protein/10^6 cells (mean ± one standard deviation of nine determinations). Values in parentheses represent the number of preparations assayed.

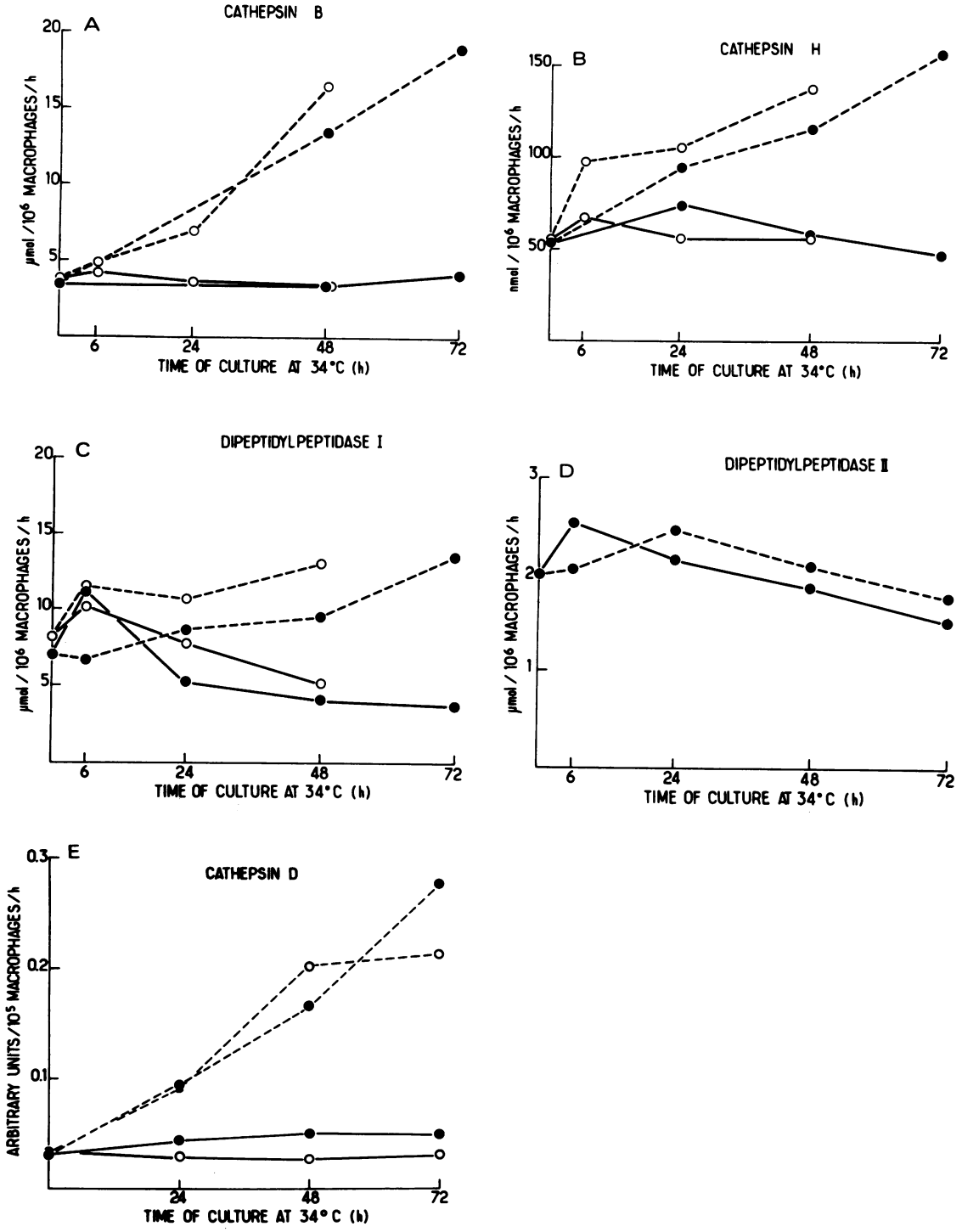

FIG. 2. Determination of protease activities associated with uninfected and infected macrophages. After 6, 24, 48, or 72 h of culture at 34°C, uninfected (——) and infected (-----) macrophages were lysed and cathepsin B (A), cathepsin H (B), DPP I (C), DPP II (D), and cathepsin D (E) activities were titrated as described in Materials and Methods. Results of one (panel D [●]) or two different (panels A, B, C, and E [●, ○]) experiments are shown and expressed as nanomoles or micromoles of reaction product per 10^6 macrophages per hour (panels A through D) or as arbitrary units per 10^6 macrophages per hour (panel E).

(Fig. 1B) whereas in larger PV, staining was located mainly on the inner aspect of their membrane (data not shown). Intravacuolar amastigotes were also stained, and this was particularly striking in large PV labeled at the periphery. Similar variations of the staining pattern with PV size have also been noted for the immunocytochemical localization of other soluble lysosomal markers (Antoine et al., unpublished results). We suspect that the pattern of antigen localization observed in large PV is due to the low concentration of internal components. These would be poorly cross-linked by

TABLE 2. Inhibition of protease activities in macrophage lysates by cysteine-, serine- or aspartic-protease inhibitors[a]

Enzyme activity	Inhibitor (concn)	% Inhibition in[b]: Uninfected macrophages	% Inhibition in[b]: Infected macrophages
Cathepsin B	Iodoacetamide (5 mM)	97.9 ± 0.9 (3)	98.2 ± 0.2 (2)
Cathepsin H	Iodoacetamide (5 mM)	87.6 ± 6.4 (2)	92.8 ± 7.1 (2)
Cathepsin H	Leupeptin (1 μM)	5.9 ± 2.7 (2)	9.5 (1)
DPP I	Iodoacetamide (5 mM)	92.7 ± 4.1 (4)	90.4 ± 4.6 (3)
DPP II	PMSF (8 mM)	77.5 ± 0.1 (2)	81.5 ± 7.0 (4)
Cathepsin D	Pepstatin (10 μM)	95.3 ± 4.8 (9)	94.0 ± 2.8 (7)

[a] Uninfected and infected macrophages were lysed after 6 to 72 h of culture at 34°C. Lysate-associated protease activities were measured with or without inhibitors.

[b] Percentages of protease activity inhibition obtained with iodoacetamide, leupeptin, pepstatin, or phenylmethylsulfonyl fluoride (PMSF) were calculated according to the formula [(specific activity without inhibitor − specific activity with inhibitor)/specific activity without inhibitor] × 100. Results are expressed as mean ± one standard deviation. Values in parentheses indicate the number of lysates tested.

paraformaldehyde and therefore poorly retained within permeabilized cells during the processing for microscopy. Only lysosomal enzymes linked through the fixative to PV membrane components or amastigotes would be retained.

Controls, which included cell preparations incubated with either conjugate alone or anti-OVA antibodies and conjugate, showed either no staining or a weak diffuse staining. Moreover, specific staining on isolated amastigotes was not detected, except with anti-cathepsin B IgG, which weakly stained megasomes (acidic lysosomelike organelles rich in hydrolases).

Protease assays. In a first series of experiments the protease activities detected in uninfected macrophages and in isolated amastigotes were compared. Results presented in Table 1 show that although the thiol-proteases cathepsin B, cathepsin H, and DPP I were present in both amastigotes and macrophages, their activities were much greater in the latter. On the other hand, seryl-protease DPP II activity was undetected in amastigotes but strongly expressed in macrophages. Finally, the aspartyl-protease cathepsin D was detected in macrophages and parasites with a similar specific activity (arbitrary units per milligram of protein per hour).

When uninfected macrophages were placed at 34°C, cathepsin B, H, and D activities remained nearly constant for up to 72 h (Fig. 2A, B, and E), whereas DPP II activity decreased very slightly over this period (Fig. 2D). A greater decrease, reaching 40% at 72 h, was noted for DPP I activity (Fig. 2C). After infection, cathepsin B and H, DPP I, and cathepsin D activities rose progressively (Fig. 2A, B, C, and E). When expressed as the percentage of control values obtained with parallel cultures of uninfected macrophages, these increases reached 358, 234, 270, and 529%, respectively, at 72 h postinfection. In contrast, DPP II activity was very similar in uninfected and infected macrophages throughout the experiment (Fig. 2D).

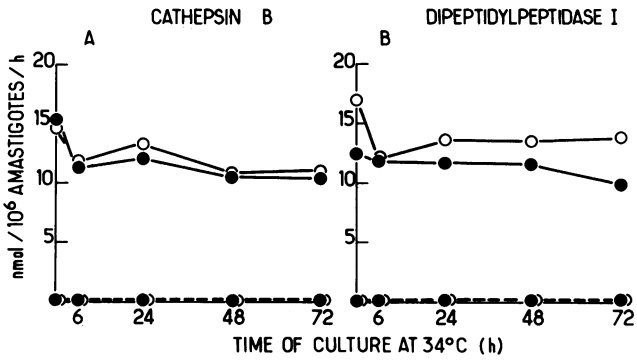

FIG. 3. Cathepsin B and DPP I activities released by and associated with isolated *L. amazonensis* amastigotes. Purified parasites were cultured in RPMI 1640-fetal bovine serum buffered at either pH 7.3 (○) or pH 5.3 (●). After various periods, samples of cell suspensions were withdrawn. Cathepsin B (A) and DPP I (B) activities in amastigote lysates (——) and in cell supernatants (-----) were determined. Results of one experiment are shown and expressed as nanomoles of released MNA per 10^6 amastigotes per hour at 37°C.

To check the enzyme specificity of biochemical assays performed on uninfected and infected macrophage lysates, we used class-specific inhibitors. Results presented in Table 2 show that in both cells, similar strong inhibition of cathepsin B, cathepsin H, and DPP I activities were obtained with the thiol-blocking reagent iodoacetamide and that almost all the cathepsin H activity was unaffected by leupeptin, an inhibitor known to be much more potent for cathepsin B than for cathepsin H (35). Likewise, DPP II activity titrated in both uninfected and infected cells was strongly inhibited by phenylmethylsulfonyl fluoride, a specific inhibitor of seryl-proteases, and cathepsin D activity was almost completely abolished by pepstatin, a specific carboxyl-protease reagent.

It is very likely that most of the protease activities titrated in infected macrophages were of host cell origin. Indeed, in infected cells, the proteolytic activities contributed by amastigotes were calculated from the values presented in Table 1 and from the number of amastigotes counted in infected cultures. They did not exceed 1, 3, 1, or 10% for cathepsin B, cathepsin H, DPP I, and cathepsin D, respectively, at 72 h postinfection. Furthermore, no evidence was obtained for the release of protease activities by amastigotes (Fig. 3). In these experiments, isolated parasites were maintained in medium buffered at pH 7.3 or 5.3, the latter being chosen because, normally, *Leishmania* amastigotes lodge within acidified phagolysosomes. Under these conditions, the viability of amastigotes tested by reduction of MTT did not change over the 72-h culture period (data not shown) and amastigote-associated cathepsin B and DPP I activities decreased only slightly. Over the same period, no enzyme activities could be detected in the media.

In situ degradation of exogenous proteins. To further assess the functional state of lysosomal proteases in *Leishmania*-infected macrophages, we incubated cells for 2 to 3 h with the endocytic tracers [^{125}I]BSA or native OVA before measuring their degradation. It was shown in preliminary experiments that during this incubation period, fluid-phase endocytic tracers reached PV (data not shown), where

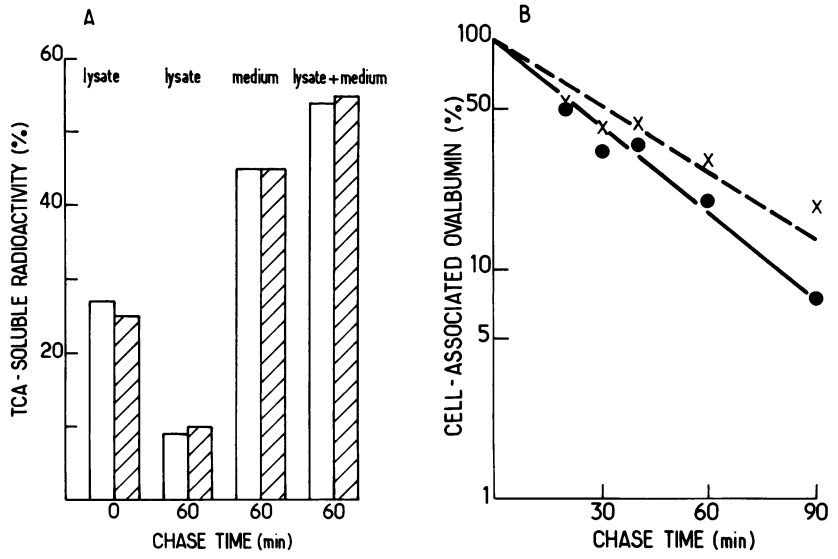

FIG. 4. Effect of infection on macrophage degradation capacity toward exogenous proteins. (A) Infected (▨) and uninfected (□) macrophages were incubated for 2 h with [^{125}I]BSA. After being washed, the cells were lysed immediately or chased in fresh medium for 60 min. Trichloroacetic acid-soluble radioactivity present in medium and cell lysates was then determined. Results are expressed as the percentage of the total radioactivity associated with unchased cells. Before the chase, uptake of BSA by uninfected and infected macrophages was 12,500 and 13,500 cpm/10^6 cells, respectively. (B) Infected (-----) and uninfected (——) macrophages were incubated for 3 h with 5 mg of OVA per ml, washed, and chased for 20 to 90 min. Cell-associated OVA was titrated by an enzyme immunoassay. Results (from two independent experiments) are expressed as percentage of OVA associated with unchased cells. Before the chase, uptake of OVA by uninfected and infected macrophages was 6.0 and 6.7 μg/10^6 cells, respectively.

exogenous protein degradation must take place, at least in part and that uptake of both [^{125}I]BSA and OVA was unmodified after infection, suggesting that the endocytic activity of infected macrophages is not grossly affected. Degradation of [^{125}I]BSA, as measured by the appearance of trichloroacetic acid-soluble radioactivity in cells and medium, was identical in uninfected and infected cells (Fig. 4A). Likewise, the degradation capacity of macrophages toward native OVA was not altered by infection, since cell-associated OVA disappeared with very similar half-lives of 24 and 31 min in uninfected and infected cells, respectively.

DISCUSSION

We provide evidence based on the use of immunocytochemical methods that, after infection of macrophages by *Leishmania* amastigotes, lysosomal proteases are translocated from secondary lysosomes to PV and surround the parasites. This conclusion is supported by the following observations. (i) In uninfected macrophages, lysosomal cathepsins were distributed in a vesicular pattern and were concentrated mainly in the perinuclear area. A very similar immunocytochemical localization of the classical lysosomal markers acid phosphatase and arylsulfatase has been reported previously (30, 39), suggesting that cathepsin-containing vesicles are, for the most part, secondary lysosomes. (ii) After infection, an almost complete depletion of these vesicles was observed, and cathepsins were then detected within PV. This probably arises from extensive fusion of secondary lysosomes with amastigote-containing phagosomes. These data agree with and complement earlier results obtained by using other acid hydrolases (4) or exogenous lysosomal tracers as indicators of fusion (1, 16, 32). (iii) The lack of reactivity of anti-rat cathepsin immunoglobulin on Western blots of amastigote lysates, as well as the negative or weak immunocytochemical staining of isolated amastigotes by these reagents, indicated that proteases detected within PV were mainly of host cell origin.

Investigations of the functional activities of five lysosomal proteases classified as endopeptidases (cathepsins B, H, and D) or exopeptidases (DPP I and II) were also performed by using specific synthetic substrates and/or specific assay conditions (8, 20, 34, 35). Whereas protease activities remained stable or slightly decreased in uninfected macrophages, four of the five activities tested increased after infection. Furthermore, the present study indicates that parasites did not make an important contribution to protease activities titrated in infected macrophages. These data are thus consistent with previous results from our laboratory which showed that acid phosphatase, arylsulfatase, and trimetaphosphatase, mainly of host cell origin, are detected within PV and exhibit unmodified or increased activity after infection (4).

The above results demonstrate that lysosomal proteases of infected macrophages are fully active in lysates which have been adjusted to conditions optimal for acid hydrolases. Such conditions are probably operative in PV, since recent findings indicate that these compartments maintain a pH at least as low as the internal pH of uninfected macrophage secondary lysosomes (4a, 14; Rivas and Chang, Abstr. MBL Gen. Meet., Biol. Bull. 1983). Furthermore, catabolism of BSA and OVA, both known to be good substrates for cysteyl- and aspartyl-proteases of the endocytic compartments (13, 18, 24), occurred at a very similar rate in uninfected and 48 h-infected macrophages. This implies that *Leishmania* infection does not interfere with the overall protein degradation capacity of the host cells and that lysosomal proteases are active in situ. However, unexpectedly, rates of degradation measured in infected macrophages did not correlate with the cysteyl-protease and cathepsin D

activities, which, 48 h after infection, were increased two- to fourfold. To explain this discrepancy we favor the hypothesis that the rise of protease activities would be offset by the huge volume increase of the lysosomal compartment following infection by *Leishmania* amastigotes of the *mexicana* complex (Antoine et al., unpublished results) and by the consequent enzyme dilution. However, we can rule out neither an inhibitory mechanism of host lysosomal enzymes requiring the participation of living amastigotes nor the presence within PV of very low-affinity inhibitors of lysosomal enzymes whose action would be removed after lysis of cells.

Overall, these results suggest that survival of intracellular *Leishmania* within phagolysosomes is not caused by inhibition of production and/or activity of lysosomal enzymes. On the contrary, *Leishmania* seem to stimulate the lysosomal enzyme machinery of the macrophage. Three kinds of nonexclusive mechanisms could account for the positive regulation observed during infection in our experimental model. These are (i) an enhanced enzyme synthesis, which has already been demonstrated as the main mechanism underlying the increase of lysosomal enzyme activities in macrophages subjected to various endocytic stimuli (7, 27); (ii) a reduced enzyme degradation, which might result from the volume increase of the lysosomal compartment (as mentioned above, this phenomenon must be accompanied by reduction of the concentration of lysosomal internal components including that of lysosomal hydrolases, which most probably are involved in their own degradation); and (iii) modulation of acid hydrolase activities by changes in endogenous inhibitors (for a review, see reference 10). However, recent data from our laboratory showing that *Leishmania* infection leads to an increase in the amount of cathepsins B and D produced by the host cells tend to support the first two explanations (data not shown).

Adaptation of amastigotes to conditions rich in protease activities might be linked to the presence on their plasma membrane of proteins resistant to proteolysis (15, 17, 23, 25). Alternatively, membrane proteins could be protected against enzymatic attack by molecules resistant to lysosomal enzymes. The lipophosphoglycan, a major cell surface glycoconjugate of *Leishmania* species (38), is a good candidate for this role. Indeed, it has been recently demonstrated that the rate of degradation of erythrocytes phagocytized by macrophages is significantly lowered after they have been coated with excreted factor, a soluble form of lipophosphoglycan (19).

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