DESTRUCTION OF *LEISHMANIA MEXICANA AMAZONENSIS* AMASTIGOTES WITHIN MACROPHAGES BY LYSOSOMOTROPIC AMINO ACID ESTERS

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*Leishmania* are insect-borne Protozoan parasites responsible for significant human mortality and morbidity in tropical and subtropical regions of the world. Different species of the parasites induce a wide spectrum of cutaneous, mucocutaneous, and visceral disease (1). In the mammalian host, the incompletely flagellated forms of the parasite, known as amastigotes, are unable to replicate extracellularly and almost exclusively infect mononuclear phagocytes (2). Amastigotes, taken in by phagocytosis, survive and multiply within membrane-bounded vacuoles (parasitophorous vacuoles; p.v.), which have at least two features in common with phagolysosomes. First, they fuse with secondary lysosomes, as shown in studies with macrophages loaded with electron-opaque tracers or with ligands recognized by mannose receptors (3–6). Second, the pH within the p.v. is acidic, at least for the *mexicana* species. While measurements in single cells are not available, this acidification is supported by the efficient concentration of weak bases such as neutral red, phenazine methosulfate, or acridine orange in the vacuoles (7, and unpublished observations by D. Biegel, F. Maxfield, and M. Rabinovitch). Such concentration is best explained by trapping through protonation (8, 9). A third expected feature of phagolysosomes, i.e., the presence of lysosomal enzyme activity in p.v. has only rarely been observed in the course of cytochemical studies (10–15). It is not known whether this lack of cytochemical reactivity reflects the inhibition or destruction of host cell lysosomal enzymes by parasite products, or the inability of the lead-capture methods used to detect the enzyme activity in the vacuolar lumen (6).

Esters of several L-amino acids have been shown (14) to rapidly disrupt rat liver lysosomal preparations, releasing lysosomal enzyme activity into the medium and reducing the turbidity of the lysosomal suspensions. Esters of D-amino acids, which are more slowly hydrolyzed, were less active (14). The mechanism proposed involved permeation of the ester into the lysosomes and its fast enzymatic hydrolysis. Since free amino acids do not readily traverse the lysosomal mem-

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**Abbreviations used in this paper:** MES, 2-(N-morpholino)ethane sulfonic acid; MOPS, 3-(N-morpholino)ethane sulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; p.v., parasitophorous vacuoles.
brane, their rapid accumulation would result in osmotic lysis of the organelles (14). This mechanism was supported in studies (15) with labeled esters in which rates of amino acid efflux from lysosomal fractions were also determined. Ester accumulation and hydrolysis were later demonstrated (16) in lysosomes (azurophilic granules) obtained from leukocytes incubated with the compounds. Esters of several amino acids, particularly L-leucine-methyl ester (L-leu-OMe), were also shown to damage human monocytes and NK cells in vitro, and the toxicity appears related to the lysosome disrupting activity (17-20).

Since *Leishmania* amastigotes lodge within acidified vacuoles and the parasites themselves are endowed with lysosomes (21, 22), we postulated that amino acid esters accumulated in the p.v. could be further concentrated in the amastigote lysosomes, leading to damage to the parasites. In this report, we show that certain amino acid esters can destroy intracellular amastigotes at concentrations that do not appear to damage the host cells. The activity depends both on the amino acid and on the ester substitution. Furthermore, since the products of ester hydrolysis are not toxic to isolated amastigotes, but the amastigotes are killed by the esters, we propose that penetration of the *Leishmania* by the esters underlies the destruction of the intracellular parasites.

**Materials and Methods**

**Animals.** Male and female BALB/c mice, 20–25 g body wt, were obtained from the Central Animal Facility, Institut Pasteur.

**Chemicals.** Unless otherwise indicated, amino acids, amino acid esters, biological buffers, and other compounds were purchased from Sigma Chemical Co., St. Louis, MO. d-Leu-OMe was obtained from Serva, Heidelberg, Federal Republic of Germany. L-leu-L-leu-OMe was from Bachem AG, Bubendorf, Switzerland. Methyl esters were mono- or dihydrochlorides and benzyl esters were obtained as p-toluene sulfonate salts. Purity of the esters was assessed by chromatography on silica gel 60 TLC plates (Merck, Darmstadt, Federal Republic of Germany) developed with 9.5:0.5 chloroform/methanol or with 3:1:1 n-butanol/acetic acid/H2O. Spots were detected with ninhydrin. Comparison of ester spots with amino acid standards indicated that the ester preparations contained 5.0–10% free amino acid. Stock solutions of the esters, prepared in HBSS at 10–100 mM and neutralized, were found to be stable for several weeks at −20°C. Stock solutions of L-leu-Obenzyl ester (250 mM), were prepared in ethanol.

**Media.** Ca++-Mg++-free PBS contained 6.7 mM potassium phosphate buffer and 138 mM NaCl, and was adjusted to pH 7.3. HBSS, DMEM, and FCS were obtained from Gibco Laboratories, Grand Island, NY. Macrophages and parasites were washed with HBSS buffered with 10 mM Hepes to pH 7.3. HBSS-Hepes-3% FBS was used in the incubation of macrophages with the esters. In studies of the pH dependence of amastigote destruction, HBSS was buffered with 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) for pHs around 5.0, or with 25 mM 3-(N-morpholino)ethanesulfonic (MOPS) for pHs in the region of 6.0–7.4. Macrophage culture medium was prepared with DMEM, with the addition of 10% FCS and 20 μg/ml gentamycin. Promastigote culture medium contained DMEM with 10% FBS, 2 mM glutamine, 10 mM Hepes, 20 μg/ml gentamycin, and 25 μg/ml 5-fluorocytosine.

**Leishmania Strain and Preparation of Amastigote Inoculum.** *Leishmania mexicana amazonsis* LV 79, originally from the Department of Parasitology, Liverpool School of Tropical Medicine, United Kingdom, was obtained in 1980 from Dr. J. P. Dedet (Institut Pasteur, Cayenne, French Guiana), passaged for 2 yr in hamsters, and thereafter in BALB/c mice. Transfers were performed by s.c. injection of 10⁸ amastigotes or cultured promastigotes into the rump of the hosts. After 4–8 wk the lesions were excised, minced in HBSS-Hepes, and disrupted by hand in a glass homogenizer. Tissue debris were
removed by centrifugation at 25 g for 10 min, and the supernatant containing most of the amastigotes was subjected to two cycles of 10 min centrifugation at 250 g. The final sediment was resuspended in HBSS-Hepes containing 3% FBS, and the number of amastigotes was determined in a hemocytometer. Promastigote cultures were established from lesion amastigotes seeded in promastigote medium and kept at 25°C.

**Macrophage Cultures.** Mouse resident or periodate-elicited peritoneal macrophages were collected in PBS and allowed to adhere to 12-mm-diam glass coverslips (~10⁶ per coverslip). The macrophage-bearing coverslips were rinsed in PBS and placed in the 16-mm-diam wells of 24-well plates (Costar, Cambridge, MA), which contained 0.5 ml macrophage culture medium per well. Cultures were kept at 34°C in a 6% CO₂ in air humidified atmosphere. Elicited macrophages were obtained 4–5 d after i.p. injection of 0.5 ml of 5 mM sodium periodate in PBS (23), and were similarly cultivated. Resident and periodate-elicited macrophages behaved alike with regard to infection and parasite destruction with the esters.

**Leishmania Infection of Macrophages.** Macrophages were infected at an estimated multiplicity of 3:1 or 5:1 amastigotes per cell in culture medium, and placed at 34°C for 24 h (24). In most instances, ≥90% of the macrophages were found to be infected.

**Treatment of Macrophages with Esters.** Pulse treatment was used in most of the experiments. Macrophages were twice washed in HBSS-Hepes, placed in HBSS-Hepes-3% FBS (pH 7.3), and after addition of esters, they were incubated for 1 h at 34°C in an air atmosphere. The cultures were washed, replaced in complete medium and returned for 16–18 h to the CO₂ incubator. At the end of this period, the cells were either fixed in methanol and stained with Giemsa (for counts of the percent infection) or fixed in 2% glutaraldehyde in PBS for phase-contrast microscopy. In other experiments, the macrophages were continuously exposed to the compounds for 16–20 h in complete medium. For the assessment of infection, ≥200 macrophages in each of two or three Giemsa-stained replicate coverslips were scored as infected or noninfected. Cells were scored as infected when they contained at least one recognizable amastigote.

**Perfusion Chamber Studies.** Macrophage cultures were prepared on 25-mm round coverslips and infected with *Leishmania*. The coverslips were mounted in Sykes-Moore chambers (Bellco Glass Inc., Vineland, NJ) filled with HBSS-Hepes-3% FBS, and placed on the stage of an inverted Diaphot-TMD Nikon (Nippon Kogaku K. K., Japan) microscope contained in a thermostatically controlled enclosure. The microscope was equipped with a Zeiss long-distance phase condenser (aperture 0.63) and Zeiss x 40 (aperture 1.0) and x 100 (aperture 1.25) oil-immersion objectives. After a suitable group of cells was selected, still 35-mm microphotographs were taken with a Nikon F2 camera back. The chamber was then perfused with HBSS containing the ester to be studied, and additional pictures taken at suitable intervals. The cultures were reperfused with ester-free HBSS and studied for additional time periods. Video recordings were made by means of a Bosch (Robert Bosch GmbH, Darmstadt, Federal Republic of Germany) model TYK 9C camera equipped with a Chalnicor tube and a Panasonic 8050 time-lapse video cassette recorder, and the images were projected on a Bosch monitor.

**Drug Treatment of Isolated Amastigotes.** Amastigote-rich suspensions were distributed in 10 x 60-mm Falcon (Falcon Labware, Oxnard, CA) tissue culture tubes, washed twice, and suspended at 2.5 x 10⁶ amastigotes/ml in appropriately buffered HBSS-3% FBS containing the compound to be studied. After 1 h at 34°C, the parasites were twice washed in HBSS-Hepes-3% FBS, and placed in promastigote medium at 26°C. At 0, 16, 24, and 48 h, samples were taken for hemocytometer counts as well for tetrazolium reduction assays (25). After adequate correlation was observed between the cell counts and the tetrazolium assays, the latter were used exclusively.

**Tetrazolium Assay.** As described by Mossman (25), 50 or 100 µl of washed (four times) parasites suspended in promastigote medium were placed in the wells of microtitre plates (Falcon Labware). To each well were added 10 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and the plates incubated for at least 4 h at 34°C. 150 µl of isopropanol containing 0.04 N HCl were then added and mixed thoroughly. After a few minutes, the plates were read at 540 nm in a Titertek Twinreader.
The results are expressed in OD units after deduction of the appropriate blanks.

Results

**Destruction of Intracellular Amastigotes by L-Leucine-methyl Ester.** Coverslip cultures of macrophages heavily infected with *L. m. amazonensis* amastigotes could be essentially cleared of parasites after pulse or continuous exposure to L-leu-OMe. In a typical experiment, resident or elicited peritoneal macrophages were infected with a 5:1 multiplicity of *Leishmania* amastigotes. The following day, >90% of the phagocytes contained one or more amastigotes, frequently lodged in the large p.v. characteristic of the *mexicana* species. Infected cultures were then pulsed with 0.8 mM L-leu-OMe for 1 h at 34°C in HBSS-Hepes-3% FBS, pH 7.3. Controls were placed in modified HBSS alone. At the end of the pulse, the cultures were washed and placed in drug-free macrophage medium for an additional 18 h. They were then fixed, stained, and the percent of infected macrophages were determined with a microscope. Under these conditions, the infection in ester-treated cultures was reduced to ≤5%. The 18-h interval between drug pulse and fixation was chosen to allow for the disposal of dead parasites, since it was difficult to distinguish viable from nonviable amastigotes in the stained preparations soon after treatment. Such pulse assays were used in most of the experiments. Alternatively, the ester was added to the complete medium for 18 h and the cultures were washed, fixed, and stained as above. Continuous exposure to the ester often required smaller concentrations for cure, but was not as experimentally versatile as pulse treatment. Fig. 1 illustrates the appearance of control cultures (infected macrophages not exposed to the ester), and of cultures treated with 1 mM L-leu-OMe for 1 h and fixed 2 or 18 h later. Control macrophages displayed numerous amastigotes in p.v. (Fig. 1A). 2 h after exposure to the ester, few if any parasites could be seen in the now apparently empty vacuoles (Fig. 1B). 18 h later, macrophages were largely free of parasites, and displayed normal morphology, although many cells carried vacuoles presumably derived from the original p.v. (Fig. 1C). Macrophage damage and reduction in cell numbers were only detected at pulse concentrations of 10 mM L-leu-OMe (not shown). The resistance of macrophages to the toxic effect of amino acid esters, in contrast to the sensitivity of monocytes was noted by others (18).

Phase-contrast microscopy of glutaraldehyde-fixed cultures proved useful in the study of early stages of parasite damage (Fig. 2). In control cultures, parasites were seen as smooth, rice-shaped bodies, usually attached to the inner surface of the p.v. (Fig. 2A). The vacuole displayed by the amastigotes is probably the flagellar pouch; within it a faintly visible flagellar root could sometimes be identified. Damage to the amastigotes could be detected as early as 20 min after exposure to the ester, as the parasites were reduced to berry-like aggregates of granules and vacuoles (Fig. 2B). Within the next hour these clusters broke up, so that granules and vacuoles were free within the lumen of the p.v. (Fig. 2C). At later times, only a few phase-dense bodies could be identified within the vacuoles (Fig. 2D).

**Studies of Living Macrophages.** Light microscopic observations of infected cultures before and after exposure to L-leu-OMe confirmed the above account.
FIGURE 1. Destruction of intracellular *Leishmania* by L-leu-OMe. Giemsa stain. (A) Control culture incubated in modified HBSS for 1 h at 34°C, then in macrophage medium for 18 h before fixation. Most of the macrophages contain amastigotes lodged in p.v. (B) Culture incubated for 1 h in medium containing 1.0 mM L-leu-OMe, washed, and placed in macrophage medium for 2 h. Macrophages display apparently empty p.v. (C) Culture treated for 1 h with 1 mM L-leu-OMe, washed, and placed in macrophage medium for 18 h. Macrophages recovered their normal appearance except for vacuoles, which may be derived from the original p.v. × 800.
of parasite destruction as seen in serially fixed cultures. The speed with which some parasites were damaged by the ester is indicated by a comparison of Fig. 3, A–C. In addition, the single-cell studies showed that the amastigotes increased in size and became rounder before they broke down (e.g., compare Fig. 3 A to C). Furthermore, the projected area of the p.v. (and probably, their volume) progressively increased during exposure of the cultures to L-leu-OMe. Most of the amastigotes initially present in the two cells illustrated in Fig. 3 were destroyed within 2.5 h of exposure to the drug. Two, however, survived, and can be seen in the last picture of the series (Fig. 3H). The effectiveness of the ester was reduced in the chamber situation when compared to treatment in the wells of tissue culture plates.

*Time and Concentration Dependence.* Parasite destruction increased with the ester concentration, and the concentration-response curves were notable for their steepness (Fig. 4). For instance, in the experiment illustrated, 87% of the macrophages exposed for 1 h to 0.5 mM of L-leu-OMe were still infected 18 h
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Figure 3. Live, infected macrophages in a Sykes-Moore chamber before (A) and after different times of treatment with 1.5 mM L-leu-OMe at 34°C (B-H). Times indicated are those after perfusion with the ester (the medium was exchanged in 1.5 min). Phase-contrast microscopy. Note the progressive enlargement of the p.x. diameters, and the swelling and sequential disruption of parasites. The time periods are: B, 10 min; C, 10.5 min; D, 20 min; E, 22 min; F, 25 min; G, 51 min; and H, 140 min × 1,500.

later, but only 10% contained parasites after a 1-h pulse with 0.75 mM concentrations. This concentration dependence in part reflects the nature of the assay, since macrophages were scored as infected whether they contained one or more parasites. Similar all or none behavior was previously found (7) in the destruction of *Leishmania* by another lysosomotropic weak base, phenazine methosulfate. Fig. 4 also shows that destruction of parasites increased with the duration of exposure of the cultures to the ester.

*Inactivity of D-Leu-OMe.* Previous studies (14, 16, 18, 20) of lysosomal disruption and cellular toxicity of amino acid esters uncovered the inactivity of the D-amino acid esters. This inactivity led to the proposal that an enzyme-catalyzed step is involved in the effects of the esters on the organelles and on the cells. Infected macrophages were subjected to pulse or continuous exposure to 5 or 10 mM concentrations of D-leu-OMe. In three separate experiments, no detect-
Figure 4. Percent of infected macrophages as a function of the concentration of L-leu-OMe and the duration of exposure of the cultures to the ester. The infection in control cultures was 95%.

Able reduction of the infection was observed. In addition, p.v. were not enlarged in these macrophages (not shown).

**Treatment of Macrophages before Infection.** Since killing of *Leishmania* can be assumed to require penetration of the ester into the p.v., we asked whether the drug would be active when given only before infection. A positive answer would indicate that the ester or its breakdown products could be efficiently transferred from preexisting lysosomes to newly formed p.v. In a typical experiment, cultures of resident macrophages were treated for 1 h with 0.8, 1.6, 4.0 or 8.0 mM L-leu-OMe. The cultures were washed twice and immediately infected with amastigotes. 18 h later, the macrophages were fixed, stained, and scored for percent infection. Controls were treated with enriched HBSS alone. No reduction in the percent infection was detected at any of the ester concentrations used. In contrast, infection was suppressed by >98% in macrophages infected for 2 or 4 h, pulsed for 1 h with 1 mM ester, and fixed 18 h later. Thus, transfer of leishmanicidal ester or ester products from lysosomes to p.v. could not be demonstrated.

**Medium pH Dependence.** The permeation of weak bases across cell membranes depends upon the concentration of the nonprotonated species in the medium (8). The latter is a function of the pK of the base and of the pH of the medium. The leishmanicidal activity of L-leu-OMe, an ester with a pK of 7.62 at 25°C (26), should be dependent on the environmental pH. Accordingly, Fig. 5 shows that higher concentrations of ester were required for leishmanicidal activity as the pH of the medium was lowered. For instance, nearly complete parasite clearing was obtained with 0.7 mM L-leu-OMe at pH 7.45, whereas parallel cultures were only incompletely cured by 2.0 mM ester at pH 6.6. That the pH effect was related to the availability of the penetrating species was suggested by a plot of the calculated concentrations of the nonprotonated species in media of the different pHs against the resulting percent of infection (not shown). A steep concentration-dependence curve was generated, similar to that obtained at a single medium pH (Fig. 4).
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**Figure 5.** Destruction of intracellular amastigotes by L-leu-OMe as a function of the medium pH and the ester concentration. Infected cultures were pulsed for 1 h at 34°C with the ester at the pHs indicated. The cultures were then washed, placed in complete macrophage medium for 18 h, and fixed.

**L-Leu-OMe Pulses at Different Temperatures.** Ester permeation through cell membranes should be rather temperature independent. In previous experiments (15, 16) accumulation of amino acid in lysosome preparations incubated with esters was faster at lower temperatures, and the proposed explanation was that ester hydrolysis was less temperature dependent than the amino acid efflux from the lysosomes. We investigated the leishmanicidal activity of L-leu-OMe in macrophage cultures pulsed for 1 h with the ester at different temperatures. The cultures were then washed and incubated in ester-free medium at 34°C for 16 h. Fig. 6 shows that L-leu-OMe was more effective in cultures pulsed at 24, 18, or 12°C (in order of increasing activity) comparison to those pulsed at 34°C. Indeed, pulses at 0°C were as effective as those at 34°C. These results confirm that ester penetration is not detectably temperature dependent. Whether reduced degradation of the ester or diminished amino acid efflux from lysosomes account for the enhanced leishmanicidal activity at lower temperatures cannot be answered by our experiments.

**Importance of Ester Group.** A few leucine esters were examined to determine the role of the ester substitution. Table I shows that the ethyl ester was two- to threefold less active than the methyl congener. Furthermore, leucine-benzyl ester (used as the tosylate) was about sixfold more active than L-leu-OMe. Sodium tosylate alone was inactive at 5 mM (not shown). The t-buty1 ester was inactive at 2 and 4 mM, with the higher concentration displaying toxicity (not shown). The only dipeptide assayed, L-leu-leu-OMe, was nearly curative at 0.08 mM.

**Esters of Other Amino Acids.** A series of esters was assayed at concentrations of up to 10 mM in at least two separate experiments involving pulse and continuous assays. Table I lists the esters in order of decreasing activity, and gives the concentrations that cured >90% of the macrophages. When only methyl esters are compared, it can be seen that the ranks are leu > trp > glu > met ≈...
Figure 6. Temperature dependence of parasite destruction by L-leu-OMe. Amastigotes were treated for 1 h at different temperatures with the indicated concentrations of the ester. The cultures were then washed and placed in macrophage medium at 34°C for 18 h before fixation.

Table 1
Amino Acid Esters Active on Intracellular Amastigotes

| Compound            | Concentration (mM) for 90% cure |
|---------------------|---------------------------------|
| L-leu-leu-OMe       | 0.08                            |
| L-leu-OBz           | 0.15                            |
| L-leu-OMe           | 0.8                             |
| L-trp-OMe           | 2.0                             |
| L-leu-OEt           | 2.5                             |
| Gly-OBz             | 3.0                             |
| L-glu-(OMe)₂        | 5.0                             |
| L-met-OMe           | 10.0                            |
| L-phe-OMe*          | 10.0                            |
| L-tyr-OMe*          | 10.0                            |

Concentrations given are derived from at least two separate experiments in which both the 1-h pulse and continuous assays were used (see Materials and Methods). D-leu-OMe, L-ile-OMe, L-val-OMe, L-alá-OMe, L-glu-OMe, L-lys-OMe, L-pro-OMe, and L-his-OMe were tested at least twice in 1-h pulse and continuous assays at 5 and 10 mM, and were found to be inactive against intracellular amastigotes. In every instance, the percent infection of the treated cultures was similar to that of controls (i.e. ≥ 80%).

* Only active in the continuous assay.

phe ≈ tyr. The last two esters were only active in the continuous assay. Methyl esters found to be inactive at 5 and 10 mM in both assays are listed in the Table I legend. It is of interest that, in contrast to L-leu-OMe, the methyl esters of L-isoleucine, and L-valine were inactive.

Effect of Amino Acid Esters and their Hydrolysis Products on Isolated Amastigotes. Destruction of Leishmania could involve the penetration of ester in the parasite; alternatively, it could be mediated by toxic ester hydrolysis products.
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liberated within the p.v. Since p.v. are most probably acidified, the effect of L-leu-OMe on isolated amastigotes was examined at pHs between 5 and 7. In a typical experiment, amastigotes prepared from lesions were washed and suspended in HBSS buffered to the desired pH with MES or MOPS, and enriched with 3% FCS. The amastigotes (2 × 10⁶ amastigotes/ml) were incubated with the compounds for 1 h at 34°C, washed, resuspended in promastigote medium, and cultured at 26°C. Viability was assessed by counting the numbers of promastigotes or determining the reduction of the tetrazolium MTT, a reduction proportional to the number of viable cells (25). Fig. 7, A and B shows that, with both kinds of assay, L-leu-OMe inhibited the transformation of amastigotes in dose-dependent fashion, and that the inhibition was markedly affected by the pH during the pulse. For instance, L-leu-OMe was quite active at 0.5 mM at pH 7.0, but at pH 6.0 and 5.0, 20- and 40-fold higher ester concentrations, respectively, were required. The effect of several esters and their hydrolysis products is shown in Table II. In these experiments, amastigotes were pulsed for 1 h with

![Figure 7](image-url)

**Figure 7.** Effect of the medium pH on the damage of isolated amastigotes by L-leu-OMe. Aliquots of a single preparation of amastigotes by L-leu-OMe. Aliquots of a single preparation of amastigotes were exposed for 1 h at 34°C to a series of concentrations of L-Leu-OMe in HBSS-FBS buffered to pH 5.0, 6.0, or 7.0. The parasites were washed and placed in promastigote medium. The viability of duplicate aliquots was determined in parallel by promastigote counts (A) or by MTT reduction (B) at 24 and 16 h, respectively, after the ester pulses. Abscissae, concentration of L-leu-OMe; ordinates, promastigotes/ml (A) or OD (B).
TABLE II
Destruction of Isolated Leishmania Amastigotes by Amino Acid Esters and their Hydrolysis Products

| Exp. | Ester or product (mM)* | MTT reduction (% ± SE) | n |
|------|------------------------|------------------------|---|
| A    | Control —              | 100 ± 2.7              | 17|
|      | L-leu-OMe (20)         | 14.6 ± 2.4             | 6 |
|      | L-leu-OBz (1)          | 19.3 ± 2.7             | 6 |
|      | D-leu-OMe (25)         | 106.0 ± 7.0            | 6 |
|      | L-ile-OMe (25)         | 96.6 ± 5.3             | 6 |
|      | L-ile-OMe (50)         | 93.0 ± 3.8             | 6 |
| B    | Control —              | 100 ± 6.3              | 4 |
|      | L-leucine (100)        | 108.4 ± 8.3            | 4 |
| C    | Control —              | 100 ± 7.1              | 4 |
|      | Methanol (100)         | 83.6 ± 2.3             | 4 |
|      | Methanol (200)         | 88.0 ± 3.6             | 4 |
|      | Benzyl alcohol (20)    | 111.8 ± 7.2            | 4 |

Viability of macrophages assayed by MTT reduction as in Materials and Methods. MTT reduction in optical density units in percentage of controls. OD values of controls were 0.150, 0.175, and 0.132, respectively in Exps. A-C.

* 1-h pulse, pH 5.0.

the compounds at pH 5.0, and incubated in promastigote medium at 25°C for 16 h before the addition of MTT for 6 h at 34°C. In parallel with the results with infected macrophages, neither L-ile-OMe nor D-leu-OMe appreciably reduced the viability of the parasites. Furthermore, L-leu-OBz, which was quite active on intracellular Leishmania at 0.15 mM (Table I), was effective on the isolated parasites at 1 mM at pH 5.0. Table II also shows that L-leucine, methanol, or benzyl alcohol were inactive when tested on the amastigotes at rather high concentrations. These results suggest that killing of intracellular Leishmania is not a consequence of the accumulation of hydrolysis products within the lumen of the p.v., but that it requires penetration of the intact ester in the amastigotes.

Discussion

Certain amino acid esters added to macrophage cultures infected with L. m. amazonensis rapidly destroyed intracellular amastigotes at concentrations that did not appear to damage the host cells. Using L-leucine-OMe, we defined some morphological features (Fig. 1–3) as well as the time and concentration dependence of the parasite destruction (Fig. 4). Parasites were not killed within macrophages exposed to the ester only before infection, implying ineffective transfer of ester or its products from preexisting lysosomes to the p.v. Amastigote destruction shares several features with those of the lysosomal and cellular effects of the compounds. (a) D-amino acid esters have low activity (14, 16, 18, 20), supporting the need for an enzyme hydrolysis step. (b) Both lysosomal accumulation (15, 16) and parasite killing by the esters are markedly affected by changes in pH and temperature. Medium pH affects the ionization of the esters, and thus
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The concentration of the nonprotonated, membrane-permeating species. For this reason, killing of the parasites by L-Leu-OMe (pK of 7.3 at 34°C) decreases as the medium pH is lowered (Figs. 5 and 7, A and B). It has been shown (15, 16) that amino acid accumulation in lysosomal preparations incubated with labeled esters reaches higher levels at 15°C than at 25 or 37°C. Likewise, the killing of intracellular *Leishmania* was maximal in cultures pulsed with L-leu-OMe at 12°C, as seen in concentration-dependence experiments (Fig. 6). The proposed explanation of the temperature effect is that amino acid efflux from lysosomes is more temperature dependent than the enzymatic degradation of the esters (15, 16). In our experiments, however, infected cultures were only kept at the lower temperature for the duration of the ester pulse. Here, it is possible that the relevant factor is the reduced rate of degradation of the ester, allowing for higher concentrations to be available to the parasites. That the esters reach the p.v. and are hydrolyzed there is indicated by the apparent enlargement of the vacuoles in the infected phagocytes (Fig. 3), an enlargement that was absent in macrophages perfused with D-leu-OMe.

We compared the leishmanicidal activity of esters of different amino acids (Table I). Of the methyl esters examined, the most active was that of leucine, which cured >90% of the macrophages at 0.8 mM, followed by tryptophan, glutamic acid (diester), methionine, phenylalanine, and tyrosine. Methyl esters of seven other amino acids were inactive at 5.0 and 10 mM concentrations (Table I). The amino acid specificity is underlined by the inactivity of the methyl esters of two aliphatic amino acids, isoleucine and valine. Inspection of Perrin’s tables (26) does not suggest that differences in the leishmanicidal activity are related to the pK of the esters. Several of the esters, including L-leu-OMe have been shown to disrupt lysosomes or to kill human monocytes or NK cells (14–20), but their relative potency does not always correlate with their leishmanicidal activity (Table I). The reason for this is unknown. However, the dipeptide L-leu-leu-OMe was particularly effective in both the inactivation of NK cells (20) and the destruction of amastigotes.

The ester substitution was also relevant to the destruction of the parasites. Benzyl was the most active leucine ester studied, followed by the methyl and ethyl esters, in order of decreasing activity (Table I). It remains to be determined whether the structural requirements for leishmanicidal activity are related to ester permeation into cells, p.v., or *Leishmania*, to rates of ester hydrolysis by macrophage and/or parasite enzymes, or to rates of efflux of the amino acid products from the host cell or parasite vacuoles. Current studies with labeled esters may clarify these issues.

We wondered whether killing of *Leishmania* resulted from the generation of toxic ester hydrolysis products next to the parasites or requires the penetration of intact ester into the organisms. In the second case, it is possible that the esters accumulated within and disrupted the parasite lysosomes (or lysosomal equivalents), although a nonlysosomal leishmanial target for the esters cannot be excluded. As an approach to this question, we examined the destruction of isolated amastigotes by a limited number of esters, as well as by some of their hydrolysis products. The results, summarized in Table II, indicate first that the esters can act directly on the amastigotes, and second, that the hydrolysis products
are not toxic to the parasites. Cinematographic studies documented that amastigotes swell and break apart within minutes of incubation with L-leu-OMe (M. Pouchelet, V. Zilberfarb, and M. Rabinovitch, work in progress). Destruction of the isolated amastigotes was also markedly pH dependent. Thus, 20-fold higher concentrations of L-Leu-OMe were required for the killing of Leishmania at pH 5.0 (a possible pH of p.v.) than at pH 7.0 (Fig. 7, A and B). The maximum intact ester concentration in p.v. and its change in time are as yet unknown, but in isolated liver lysosomes, a 100-fold overall ester enrichment has been estimated on the basis of amino acid accumulation (15). It is thus possible that concentrations sufficient to damage Leishmania have been attained in our experiments. While it could be surmised that macrophage and parasite enzymes would be in competition for the esters, there is no direct proof that active macrophage lysosomal enzymes are present in the p.v. (6). Little is known about the nature, characteristics and substrate requirements of the enzymes responsible for ester hydrolysis in macrophage and Leishmania (27). Furthermore, at concentrations higher than those sufficient to kill the parasites, the esters can be toxic to macrophages and other cells (16–20). If the enzymatic and other cellular mechanisms that underlie the leishmanicidal activity of the esters can be clarified, it may be possible to design esters or related compounds that can be more efficiently split by Leishmania than by the macrophage enzymes. Such molecules would be more specifically leishmanicidal and relatively less toxic to the host.

Summary

Leishmania amastigotes parasitize almost exclusively the mononuclear phagocytes of mammals. The organisms survive and multiply within acidified vacuoles (parasitophorous vacuoles; p.v.) akin to phagolysosomes. Certain amino acid esters are known to accumulate in and disrupt lysosomes. We postulated that, since Leishmania possess lysosome-like organelles, they may be susceptible to the potentially high ester concentrations attained in the p.v. We report here that L-amino acid esters can rapidly destroy intracellular Leishmania at concentrations that do not appear to damage the host cells. L-leu-OMe, which cured ≥90% of infected macrophages at 0.8 mM concentrations, was used in most of the experiments. L-leu-OMe was only active after infection, implying inefficient transfer from secondary lysosomes to the p.v. Parasite destruction had several features in common with lysosomal and leukocyte damage induced by the esters, i.e., inactivity of D-amino acid esters, a marked pH dependence and increased killing after ester pulses at lower temperatures. Killing depended on the amino acid and on the ester substitution. The most active of the methyl esters assayed was that of leucine, followed by those of tryptophan, glutamic acid, methionine, phenylalanine, and tyrosine. Methyl esters of seven other amino acids were inactive when tested at up to 10 mM concentrations. Among leucine esters studied, benzyl ester was sixfold more active than the methyl homolog. The dipeptide L-leu-leu-OMe produced 90% cure at 0.08 mM concentrations. Leishmanicidal activity could be related to penetration of the parasites by the esters or to toxic ester hydrolysis products released in the p.v. The first hypothesis is supported by the pH-dependent destruction of isolated amastigotes by the esters. Furthermore, relatively high concentrations of L-leucine, methanol, or benzyl
alcohol were not demonstrably toxic to the amastigotes. We postulate that ester concentrations sufficient to damage the intracellular amastigotes may be obtained within the p.v. after exposure of infected macrophages to the esters. Esters preferentially hydrolyzed by parasite enzymes may be expected to be leishmanicidal, but less damaging to the host.

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References
1. Zuckerman, A., and R. Lainson. 1977. Leishmania. In Parasitic Protozoa, vol. I. J. P. Kreier, editor. Academic Press, New York. 57–133.
2. Mauel, J., and R. Behin. 1982. Leishmaniasis: immunity, immunopathology and immunodiagnosis. In Immunology of Parasitic Protozoa. S. Cohen and K. S. Warren, editors. Blackwell Press, Oxford, United Kingdom. 299–355.
3. Chang, K. P. 1983. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int. Rev. Cytol. 14 (Suppl.): 267.
4. Shepherd, V. L., P. D. Stahl, P. Bernd, and M. Rabinovitch. 1983. Receptor-mediated entry of β-glucuronidase into the parasitophorous vacuoles of macrophages infected with Leishmania mexicana amazonensis. J. Exp. Med. 157:1471.
5. Rabinovitch, M., G. Topper, P. Cristello, and A. Rich. 1985. Receptor-mediated entry of peroxidases into the parasitophorous vacuoles of macrophages infected with Leishmania mexicana amazonensis. J. Leukocyte Biol. 37:247.
6. Rabinovitch, M. 1985. The endocytic system of Leishmania-infected macrophages. In Mononuclear Phagocytes. Characteristics, Physiology and Function. R. van Furth, editor. Martinus Nijhoff, Dordrecht, The Netherlands. pp. 611–619.
7. Rabinovitch, M., J. P. Dedet, A. Ryter, R. Robineaux, G. Topper, and E. Brunet. 1982. Destruction of Leishmania mexicana amazonensis amastigotes within macrophages in culture by phenazine methosulfate and other electron carriers. J. Exp. Med. 155:415.
8. de Duve, C., T. de Barsy, B. Poole, A. Trouet, P. Tulkens, and F. van Hoof. 1974. Lysosomotropic agents. Biochem. Pharmacol. 23:2495.
9. Dean, R. T., W. Jessup, and C. R. Robert. 1984. Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. Biochem. j. 217:27.
10. Alexander, J., and K. Vickerman. 1975. Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of Leishmania mexicana-infected macrophages. J. Protozool. 22:502.
11. Lewis, D. H., and W. Peters. 1977. The resistance of intracellular Leishmania parasites to digestion by lysosomal enzymes. Ann. Trop. Med. Parasitol. 71:295.
12. Ryter, A., J. P. Dedet, and M. Rabinovitch. 1983. Leishmania mexicana: acid phosphatase ultrastructural cytochemistry of infected mouse macrophage cultures treated with phenazine methosulfate. Exp. Parasitol. 55:233.
13. Barbieri, C., K. Brown, and M. Rabinovitch. 1985. Depletion of secondary lysosomes in mouse macrophages infected with Leishmania mexicana amazonensis: a cytochemical study. Z. Parasitenkd. 71:159.
14. Goldman, R., and A. Kaplan. 1973. Rupture of rat liver lysosomes mediated by L-aminoacid esters. Biochim. Biophys. Acta. 318:205.
15. Reeves, J. P. 1979. Accumulation of amino acids by lysosomes incubated with amino acid methyl esters. J. Biol. Chem. 254:8914.
16. Ransom, J. T., and J. P. Reeves. 1983. Accumulation of amino acids within intracellular lysosomes of rat polymorphonuclear leukocytes incubated with amino acid methyl esters. J. Biol. Chem. 258:9270.

17. Thiele, D. L., L. Kurosaka, and P. E. Lipsky. 1983. Phenotype of the accessory cell necessary for mitogen-stimulated T and B responses in human peripheral blood: delineation of its sensitivity to the lysosomotropic agent L-leucine methyl ester. J. Immunol. 131:2282.

18. Thiele, D. L., and P. E. Lipsky. 1985. Modulation of human natural killer cell function by L-leucine methyl ester: monocyte dependent depletion from human peripheral blood mononuclear cells. J. Immunol. 134:786.

19. Shau, H., and S. H. Golub. 1985. Depletion of NK cells with the lysosomotropic agent L-leucine methyl ester and the in vitro generation of NK activity from NK precursor cells. J. Immunol. 134:1130.

20. Thiele, D. L., and P. E. Lipsky. 1985. Regulation of cellular function by products of lysosomal enzyme activity: elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes. Proc. Natl. Acad. Sci. USA. 82:2468.

21. McAlpine, J. C. 1970. Electronic cytochemical demonstration of a lysosome in Leishmania donovani. Trans. R. Soc. Trop. Med. Parasitol. 64:822.

22. Vickerman, K. 1974. The ultrastructure of pathogenic flagellates, in Trypanosomiasis and Leishmaniasis with special reference to Chagas disease. Ciba Found. Symp. 20:171.

23. Takemura, R., and Z. Werb. 1984. Regulation of elastase and plasminogen activator secretion in resident and inflammatory macrophages by receptors for the Fc domain of immunoglobulin G. J. Exp. Med. 159:152.

24. Biegel, D., G. Topper, and M. Rabinovitch. 1983. Leishmania mexicana: temperature sensitivity of isolated amastigotes and of amastigotes infecting macrophages in culture. Exp. Parasitol. 56:289.

25. Mossmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application for proliferation and cytotoxicity assays. J. Immunol. Methods. 65:55.

26. Perrin, D. D. 1972. Dissociation constants of organic bases in aqueous solution: Supplement 1972. Butterworths, London. pp. 375–403.

27. Barrett, A. J., and M. F. Heath. 1977. Lysozymes. In Lysosomes: A laboratory handbook. J. T. Dingle, editor. North/Elsevier Holland, Amsterdam The Netherlands. p. 30.