INFECTION AND IMMUNITY, Mar. 1990, p. 779–787
0019-9567/90/030779-09$02.00/0

Vol. 58, No. 3

# Parasitophorous Vacuoles of *Leishmania amazonensis*-Infected Macrophages Maintain an Acidic pH

JEAN-CLAUDE ANTOINE,[1]* ERIC PRINA,[1] COLETTE JOUANNE,[1] AND PIERRE BONGRAND[2]

*Département de Physiopathologie expérimentale, Unité d' Immunophysiologie cellulaire de l' Institut Pasteur et du CNRS (UA 1113), 25 rue du Dr. Roux, 75724 Paris Cedex 15,[1] and Laboratoire d' Immunologie, Hôpital de Sainte-Marguerite, 13277 Marseille Cedex 09,[2] France*

Received 2 November 1989/Accepted 30 November 1989

***Leishmania* amastigotes are intracellular protozoan parasites of mononuclear phagocytes which reside within parasitophorous vacuoles of phagolysosomal origin. The pH of these compartments was studied with the aim of elucidating strategies used by these microorganisms to evade the microbicidal mechanisms of their host cells. For this purpose, rat bone marrow-derived macrophages were infected with *L. amazonensis* amastigotes. Intracellular acidic compartments were localized by using the weak base 3-(2,4-dinitroanilino)-3'-amino-*N*-methyldipropylamine as a probe. This indicator, which can be detected by light microscopy by using immunocytochemical methods, mainly accumulated in perinuclear lysosomes of uninfected cells, whereas in infected cells, it was essentially localized in parasitophorous vacuoles, which thus appeared acidified. Phagolysosomal pH was estimated quantitatively in living cells loaded with the pH-sensitive endocytic tracer fluoresceinated dextran. After a 15- to 20-h exposure, the tracer was mainly detected in perinuclear lysosomes and parasitophorous vacuoles of uninfected and infected macrophages, respectively. Fluorescence intensities were determined from digitized video images of single cells after processing and automatic subtraction of background. We found statistically different mean pH values of 5.17 to 5.48 for lysosomes and 4.74 to 5.26 for parasitophorous vacuoles. As for lysosomes of monensin-treated cells, the pH gradient of parasitophorous vacuoles collapsed after monensin was added. This very likely indicates that these vacuoles maintain an acidic internal pH by an active process. These results show that *L. amazonensis* amastigotes are acidophilic and opportunistic organisms and suggest that these intracellular parasites have evolved means for survival under these harsh conditions and have acquired plasma membrane components compatible with the environment.**

In their amastigote forms, *Leishmania* species (order, *Kinetoplastidae*; family, *Trypanosomatidae*) are obligate intracellular parasites of cells belonging to the monocytic lineage.They enter host cells by phagocytosis and multiply within organelles of phagolysosomal origin called parasitophorous vacuoles (PV) (9, 12). Two nonexclusive hypotheses can be put forward to explain how these parasites grow in this potentially harsh environment. (i) They have accommodated to these conditions and evolved means for survival in the presence of microbicidal agents; (ii) they produce factors which inhibit or destroy toxic molecules synthesized by the macrophages. To approach this question, we looked for, in previous studies, the presence of acid hydrolases within PV. Phosphatases, sulfatases (4), and various proteases of host cell origin (E. Prina, J.-C. Antoine, B. Wiederanders, and H. Kirschke, manuscript in preparation) have been detected in these compartments by cytochemistry or immunocytochemistry. Furthermore, the activities of these enzymes, assayed biochemically in cell extracts, are unaffected or increase after infection, suggesting that *Leishmania* parasites do not reduce the amount or the activity of host lysosomal enzymes. These results are thus in agreement with an accommodation of *Leishmania* parasites to hydrolytic conditions. However, nothing is known about the in situ activity of lysosomal enzymes. In this respect, the phagolysosomal pH is a key parameter which controls the activity of lysosomal enzymes (7), as well as other microbicidal mechanisms such as the formation of reduced oxygen species (30, 31). Until now, only a few preliminary studies have addressed the issue of PV pH. Moreover, they have yielded contradictory results. Thus, some researchers claim that the internal milieu of PV is as acidic as that of secondary lysosomes of uninfected macrophages (11; L. Rivas and K.-P. Chang, Biol. Bull. **165**:536, 1983), whereas others find an almost neutral pH (G. H. Coombs and J. Alexander, cited in reference 9).

The purpose of the present study was to reassess this debated point. Acidic compartments present in *Leishmania amazonensis*-infected macrophages were characterized by using 3-(2,4-dinitroanilino)-3'-amino-*N*-methyldipropylamine (DAMP) as a probe (1). This weak base can be detected by immunocytochemistry with antidinitrophenol (anti-DNP) antibodies and has been previously used to localize acidic organelles in several different types of cells (2). This qualitative approach was completed by pH quantitations performed on single cells whose secondary lysosomes or PV had been previously filled with the pH probe fluorescein-labeled dextran (F-Dex). This method was introduced by Ohkuma and Poole (39) and is based on measuring the pH-dependent fluorescence intensity ratio with blue- and purple-wavelength excitation. Results obtained show that PV are strongly acidified, which supports the hypothesis that *Leishmania* species are truly adapted to at least some phagolysosomal conditions.

## MATERIALS AND METHODS

**Animals.** Female BALB/c mice 2 to 4 months old and 3- to 5-month-old male Fischer 344 rats were obtained from the breeding center of the Pasteur Institute or from Iffa Credo (St-Germain-sur-l'Arbresle, France).

**Preparation of amastigotes.** *L. amazonensis* LV79 (World Health Organization reference number MPRO/BR/72:

* Corresponding author.

M1841) was propagated in BALB/c mice by subcutaneous injection of $10^6$ amastigotes into each hind footpad; 2 to 5 months later, lesions were excised and amastigotes were prepared as described previously (3). Before infection of macrophage cultures, parasites were separated from host cell debris by centrifugation on a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) (10).

**Macrophage cultures.** Bone marrow cells were flushed from femurs and tibias of Fischer rats and distributed in 100-mm tissue culture dishes ($1.6 \times 10^7$ cells per dish; Corning Glass Works, Corning, N.Y.) containing 16 ml of Dulbecco modified Eagle minimal essential medium (Seromed, Berlin, Federal Republic of Germany) or RPMI 1640 medium (GIBCO Laboratories, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum, 10% L cell-conditioned medium, and 50 μg of gentamicin (Sigma Chemical Co., St Louis, Mo.) per ml. Cells were cultured at 37°C in a humidified 6% (Dulbecco medium) or 5% (RPMI medium) $CO_2$ in 94 or 95% air atmosphere. After 5 days, adherent macrophages were harvested as described earlier (4) and allowed to attach onto 12-mm-diameter glass cover slips ($3 \times 10^4$ to $1.2 \times 10^5$ cells per cover slip) or 35-mm petri dishes (Corning) ($1 \times 10^6$ cells per dish). Cells were cultured as previously described (4) and used after 24 h at 37°C.

**Infection of macrophage cultures.** Amastigotes were added to macrophage cultures at a multiplicity of four parasites per host cell. Control and infected cultures were placed at 34°C for 6, 24, or 48 h in a humidified 5 or 6% $CO_2$ in 94 or 95% air atmosphere.

**Assessment of degree of infection of macrophage cultures.** To estimate macrophage numbers, we lysed cells in petri dishes with 50 mM Tris hydrochloride buffer (pH 7.5) containing 25 mM KCl, 10 mM $MgCl_2$, and 0.5% (vol/vol) Nonidet P-40 (Sigma). After 10 min at room temperature and 20 min at 4°C, only macrophage nuclei remained intact, and they were counted in a hemacytometer. The number of viable parasites present in infected cultures was estimated as described earlier (4) after lysis of macrophages in petri dishes with 0.005% sodium dodecyl sulfate in Hanks medium (Diagnostics Pasteur, Marnes-la-Coquette, France) buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Eurobio, Paris, France).

To estimate percentages of infected macrophages, we fixed cells on cover slips with 2% glutaraldehyde (Sigma grade I) in 0.05 M cacodylate hydrochloride buffer (pH 7.2) for 1 h at room temperature. Infected macrophages were scored by phase-contrast microscopy, and percentages were determined after counting about 1,000 cells per preparation.

**Purification of antibodies and preparation of antibody-enzyme conjugates.** Anti-DNP immune serum was prepared in rabbits by injecting dinitrophenylated human immunoglobulin G mixed with complete Freund adjuvant into hind footpads. Rabbit anti-DNP antibodies were purified by affinity chromatography with Sepharose 4B (Pharmacia) coupled to DNP as the immunoadsorbent. Sheep anti-rabbit immunoglobulin and rabbit anti-hen egg ovalbumin antibodies were isolated from hyperimmune sera by using polyacrylamide-antigen immunoadsorbents (44). Sheep anti-rabbit immunoglobulin antibodies were then labeled with horseradish peroxidase (HRP grade I; Boehringer GmbH, Mannheim, Federal Republic of Germany) by a two-step glutaraldehyde coupling procedure (5).

**Incubation of macrophages with DAMP.** DAMP was synthesized by V. Huteau and J. Igolen (Unité de Chimie organique, Institut Pasteur) using a previously described procedure (1). A $5 \times 10^{-3}$ M stock solution of this compound was made in absolute ethanol and kept at −20°C. Macrophages on cover slips were incubated for 30 min at 34°C with 50 μM DAMP in RPMI medium (without $NaHCO_3$) buffered with 20 mM HEPES (Flow Laboratories, Irvine, United Kingdom) and containing 10% fetal calf serum and 50 μg of gentamicin per ml. Control cells were incubated with a concentration of ethanol equivalent to that used for DAMP-treated cells. Cell preparations were then washed twice at room temperature with Dulbecco phosphate-buffered saline (PBS) before reincubation for 5 min at 34°C. In some experiments, cells were preincubated with 5 μM monensin (sodium salt; Calbiochem-Behring, La Jolla, Calif.) or with 0.2% ethanol (controls) before adding DAMP. Stock solutions of monensin were prepared in absolute ethanol shortly before use.

**Incubation of macrophages with F-Dex.** At 8 to 9 h or 32 to 33 h after infection or the beginning of the culture at 34°C (uninfected macrophages), cells on cover slips were incubated with 1 to 2 mg of F-Dex (Sigma) per ml (average molecular weight, 35,600 to 42,000; 0.006 to 0.011 mol of fluorescein per mol of glucose). Culture at 34°C was continued for 15 to 20 h. Cells were then washed twice at room temperature with Dulbecco PBS and prepared immediately for fluorescence microscopy and fluorescence intensity measurements or chased at 34°C in RPMI-HEPES-FCS medium for various periods before observation and intensity readings.

**Immunocytochemical localization of DAMP.** After incubation with DAMP and washings, cells were fixed with 4% paraformaldehyde (Merck-Schuchardt, Darmstadt, Federal Republic of Germany) in 0.1 M sodium cacodylate hydrochloride buffer (pH 7.4) for 1 h at room temperature. Cell preparations were then quenched with 50 mM $NH_4Cl$ in PBS and permeabilized with PBS containing 0.1 mg of saponin (Sigma) per ml and 10% normal rat serum. After being washed with PBS-saponin, cover slips were sequentially incubated for 60 min at room temperature with 2.5 μg of rabbit anti-DNP antibodies per ml (or with 2.5 μg of rabbit antiovalbumin antibodies per ml for control cover slips) and with 20 μg of HRP-linked sheep anti-rabbit immunoglobulin antibodies per ml. Some control cover slips were also incubated with conjugate alone. Antibodies and conjugate were diluted in PBS containing 0.25% gelatin and 0.1 mg of saponin per ml. Peroxidase activity was detected with 3-amino-9-ethylcarbazole (Sigma) and $H_2O_2$ (Prolabo, Paris, France) as described previously (22) or with 3,3'-diaminobenzidine tetrahydrochloride (Prolabo) and $H_2O_2$ dissolved in 0.1 M Tris hydrochloride buffer (pH 7.6) (21) or in 0.05 M sodium acetate buffer (pH 5). Cell preparations were mounted in Mowiol (Calbiochem).

**Fluorescence microscopy, image digitization, and measurement of fluorescence intensity.** Fluorescence experiments were conducted on an Olympus IMT2 inverted microscope system equipped with interchangeable blue ($455 \text{ nm} < \lambda < 490 \text{ nm}$) and purple ($370 \text{ nm} < \lambda < 430 \text{ nm}$) band-pass excitation filters and with an emission filter allowing wavelengths above 510 nm to be recorded (P. André, C. Capo, A.-M. Benoliel, M. Buferne, and P. Bongrand, Cell Biophys., in press). After incubation with F-Dex, washing, and a chase, cells were covered with Dulbecco PBS containing 20 mM D-glucose and 2 mg of bovine serum albumin per ml and examined at room temperature with a planapo UV 40× objective (0.85 numerical aperture). In some experiments, monensin (20 μM) was added to the medium just before fluorescence observations or measurements. Video

images of fluorescence at blue- and purple-wavelength excitation were obtained with a high-sensitivity ($10^{-3}$ lux) video camera (model 1036; Lhesa, Cergy-Pontoise, France) mounted on the microscope. The camera output was connected to a PC vision plus digitizer (Imaging Technology, Woburn, Mass.) mounted on an IBM-compatible Zenith 148 computer. Images of 512 × 512 pixels with 256 intensity levels were obtained. Digitized fields were analyzed immediately or stored on floppy disks for delayed study. After blue or purple excitations, the total fluorescence intensity of each individual cell examined was determined and the ratio of the fluorescence intensity measured with blue excitation to that with purple excitation, referred to henceforth as the blue/purple ratio, was calculated after automatic subtraction of the extracellular background. Autofluorescence, which might have biased fluorescence values, was not taken into account for the calculations since it gave no signal with the camera sensitivity used. Subcellular analysis of labeled cells was also performed on stored digitized images with a resolution of about 1 $\mu m^2$ per pixel.

A calibration curve for determining pH values from fluorescence measurements was constructed with fixed cells equilibrated with buffers of different pH values. After incubation with F-Dex, uninfected macrophages were fixed for 30 min at room temperature with 4% paraformaldehyde in Dulbecco PBS (pH 7.4). Cells were then washed with PBS and incubated for at least 1 h at room temperature with 0.1 M citrate-phosphate buffers containing 20 μM monensin whose pH ranged between 4.5 and 7.0. Fluorescence intensities of individual cells were measured as described above. Between pH 4.5 and 7.0, fixed cells yielded blue/purple ratios very similar to those of solutions of F-Dex in the same citrate-phosphate buffers.

**Statistical analysis of results.** Data were analyzed by the Student and Fisher's *t* tests or by the nonparametric Mann-Whitney U test.

## RESULTS

**Course of amastigote infection in macrophage cultures.** Rat bone marrow-derived macrophages were highly susceptible to infection by *L. amazonensis* amastigotes (Fig. 1). Thus, from 24 h after the beginning of infection, about 80 to 85% of the macrophages contained viable parasites (Fig. 1A). Furthermore, amastigotes multiplied quite well within these host cells throughout the examined time period (Fig. 1B). Within macrophages, amastigotes were tightly bound to one side of PV, which were of relatively small size 6 h after infection but considerably enlarged later on. Thus, 24 h after adding parasites, PV occupied about 70 to 80% of the total cell volume (data not shown).

**Localization of acidic compartments in uninfected and infected macrophages.** Acidic compartments were visualized by an immunocytochemical procedure with DAMP as a pH probe. In uninfected macrophages, DAMP-containing structures appeared as HRP-positive granules distributed in the cytoplasm but much more numerous in the juxtanuclear Golgi region (Fig. 2A). A similar pattern of staining was observed whatever the time of culture at 34°C. Very likely, most of these structures represent secondary lysosomes and internal endosomes (13, 26, 35). Only a very small number of granules accumulating DAMP were located at the cytoplasmic periphery, suggesting that early endosomes known to be acidic organelles (26, 47) were unstained or weakly stained under our experimental conditions. In infected macrophages, perinuclear granules able to trap DAMP gradually

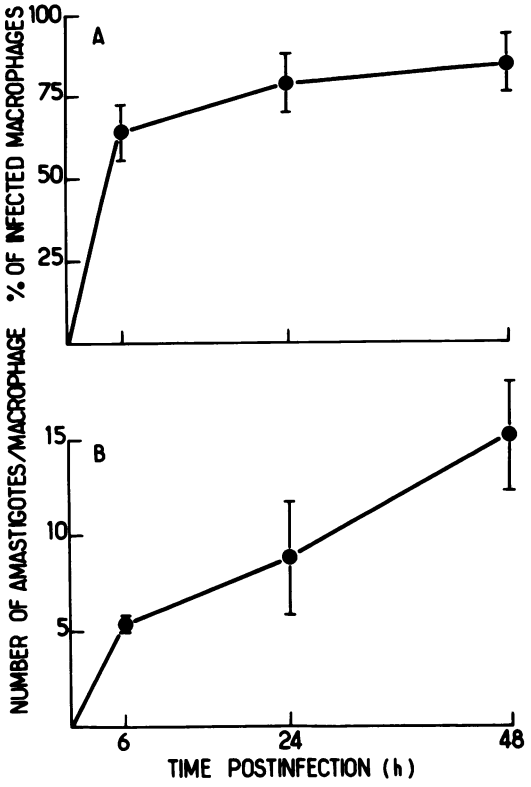


FIG. 1. Time course of infection of rat bone marrow-derived macrophages by amastigotes. Macrophages were infected at a multiplicity of four amastigotes per host cell. (A) Percentage of infected macrophages was estimated by phase-contrast microscopy at various times after adding parasites. Data points are the mean ± 1 standard deviation (SD) of 7 to 15 independent experiments. (B) Growth of amastigotes within infected cells was assessed by counting parasites released from PV after selective lysis of macrophages with Hanks medium containing 0.005% sodium dodecyl sulfate. Data points are the mean ± 1 SD of 4 to 13 independent experiments. Statistical comparisons between the different experimental groups of panel B were made by the Mann-Whitney U test and gave the following results: 6 and 24 h, $\alpha = 0.05$; 24 and 48 h, $\alpha < 0.01$.

disappeared, and 24 h after the beginning of infection, most of them had disappeared. In parallel, PV were progressively endowed with the capacity to accumulate DAMP. Thus, at 6 h after infection, only some of these organelles were positive for DAMP, but at 24 and 48 h, most if not all exhibited DAMP accumulation (Fig. 2C). The staining pattern varied according to the size of the PV. Small PV displayed intense lumenal labeling, whereas in dilated PV, DAMP was only detected at the periphery and in association with amastigotes (Fig. 2C, E, and F). The presence of DAMP in the lumen of PV or associated with the inner face of the PV membrane was confirmed by ultrastructural immunogold labeling (J.-C. Antoine and A. Ryter, unpublished data). Similar variations of the staining pattern with PV size were already noted for the immunocytochemical localization of four soluble lysosomal proteases (unpublished data). We suggest that the peculiar antigen localizations observed in large PV are due to the weak concentrations of their internal components, which thereby would be poorly cross-linked by paraformaldehyde and poorly retained within permeabilized cells during the processing for microscopy. Only DAMP or lysosomal enzymes linked through the fixative to PV membrane components or amastigotes would be retained.

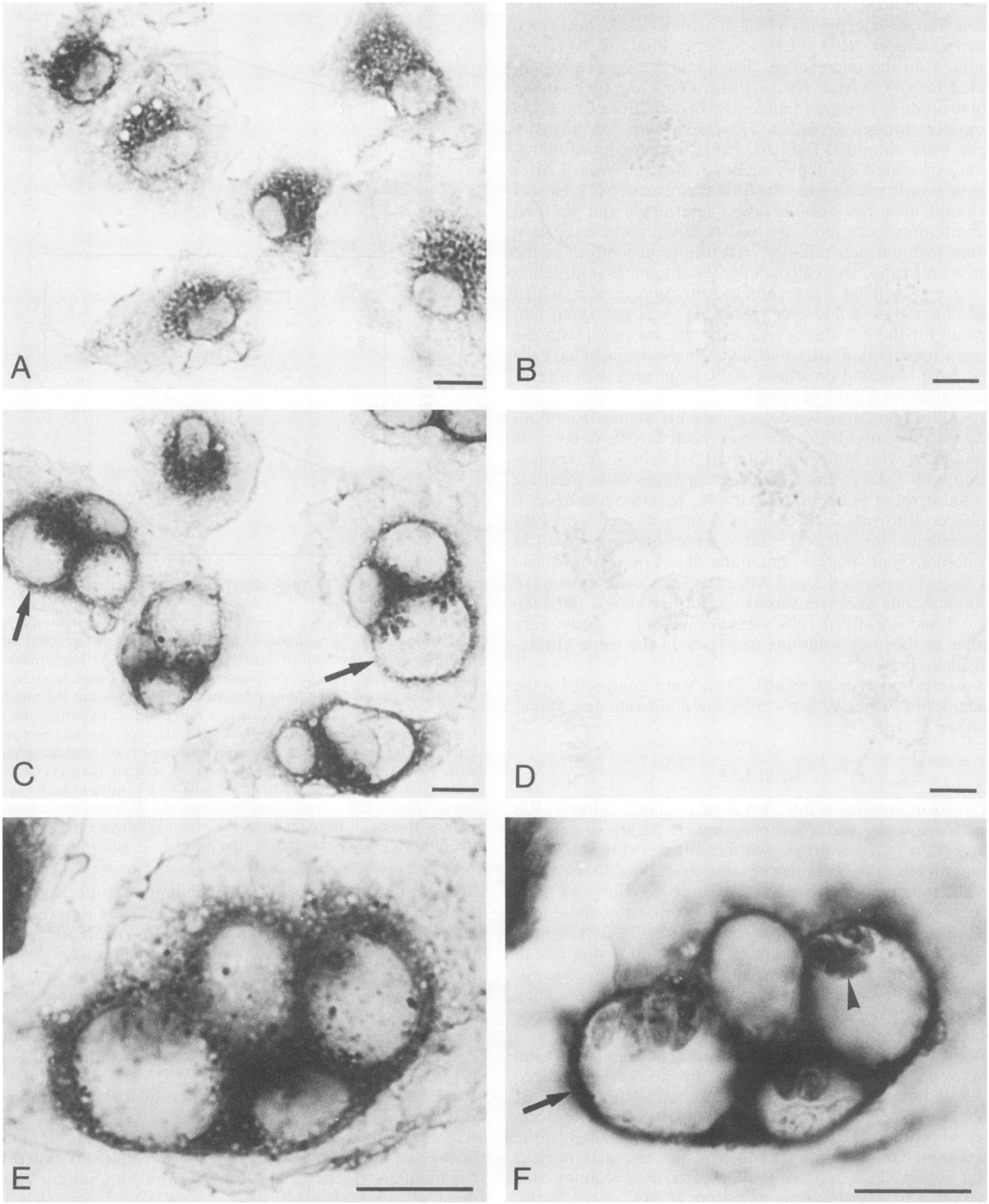


FIG. 2. Localization of sites of DAMP accumulation in uninfected (A) and infected (C, E, and F) macrophages taken 48 h after adding parasites. After incubation with DAMP, cells were fixed, permeabilized, and successively incubated with anti-DNP antibodies and HRP conjugate. As controls, DAMP-treated uninfected (B) and infected (D) macrophages were incubated after fixation and permeabilization with antiovalbumin antibodies and HRP conjugate. Cell-associated HRP activity was detected with 3,3'-diaminobenzidine and $H_2O_2$ dissolved in 0.05 M acetate buffer (pH 5). Staining appears as black perinuclear granules in uninfected cells or is associated with the periphery of PV (arrows) and with amastigotes (arrowhead) in infected cells. The same cell at two different foci is shown in panels E and F. Bars represent 10 μm.

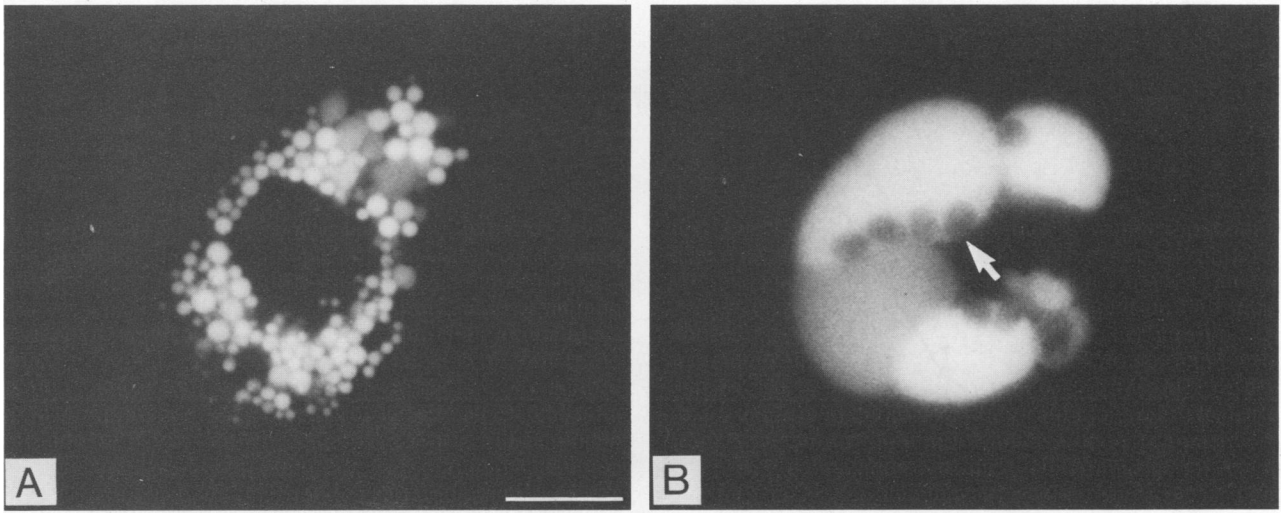


FIG. 3. Localization of F-Dex in uninfected (A) and infected (B) macrophages. Cells were photographed 26 h (A) and 29 h (B) after the beginning of the culture at 34°C or after infection and 17 to 20 h after adding F-Dex. The uninfected macrophage shows a strong labeling in perinuclear secondary lysosomes, whereas the infected macrophage exhibits labeling only within PV. Amastigotes are unstained (arrow). Bar represents 10 μm.

Control preparations, which included cells treated with ethanol instead of DAMP and DAMP-treated cells incubated after fixation with either rabbit antiovalbumin antibodies instead of anti-DNP antibodies or conjugate alone, were negative (Fig. 2B and D) except for some rare cells exhibiting endogenous peroxidase activity.

To check that DAMP accumulated within acidic compartments, we preincubated cells with 5 μM monensin, which dissipates proton gradients, before a 30-min incubation with DAMP in the continuous presence of monensin. Under these conditions, labeling of perinuclear granules and of PV was almost completely abolished (data not shown), and only a slight diffuse staining could still be observed.

**Intracellular localization of F-Dex in uninfected and infected macrophages.** The preceding experiments indicated that PV are acidic compartments. To obtain a more accurate estimation of the pH of these organelles, we incubated cells with the pH-sensitive fluid-phase endocytic tracer F-Dex. This strategy is based on two pieces of information. (i) PV have been shown to be endocytic compartments (for a review, see reference 40), and (ii) fluorescein-conjugated macromolecules internalized by endocytosis have been widely used to study the pH of intracellular compartments because of the sensitivity of both the excitation spectrum and the fluorescence intensity of fluorescein to environmental pH. A 15- to 20-h incubation time was chosen to label mainly the late endocytic compartments. Examination of living cells by conventional fluorescence microscopy showed that under these conditions, uninfected macrophages accumulated F-Dex essentially in perinuclear round granules of variable size whose pattern was typically that of secondary lysosomes (Fig. 3A). Very mobile and slightly stained small vesicles and short tubules were also observed at the cell periphery. In infected macrophages observed 24 to 53 h after amastigotes were added, strongly stained perinuclear secondary lysosomes had almost completely disappeared and in many cells F-Dex could be only detected in PV (Fig. 3B). No labeling was associated with amastigotes, which thus appeared in the PV by negative staining (Fig. 3B, arrow). Lack of parasite staining was confirmed in amastigotes isolated from F-Dex-loaded macrophages. In these preparations, most parasites were negative. Only a very few exhibited a faint staining apparently localized in the flagellar pocket (data not shown).

Addition of 20 μM monensin to the observation medium led to an immediate increase of fluorescence intensity of PV and of secondary lysosomes of uninfected macrophages, indicating that under physiological conditions, both organelles maintain an acidic internal pH.

These morphological examinations clearly indicate that in the quantitative fluorescence analysis of uninfected and infected macrophages described below, we were truly measuring the fluorescence intensity of secondary lysosomes and PV, respectively. Furthermore, they showed that amastigotes did not interfere with the pH measurement of PV in view of their almost complete absence of fluorescence.

**pH measurements of secondary lysosomes and PV. (i) Technical aspects.** In these experiments, each selected cell was sequentially excited with purple and blue wavelengths and the blue/purple fluorescence ratio was automatically calculated after digitization and processing of the video images. Ratios obtained were converted to pH values by referring to a standard curve made with fixed F-Dex-loaded macrophages equilibrated with buffers of known pH. The blue/purple ratio was strikingly pH dependent between pH 4.5 and 7.0 (Fig. 4).

Although the microscope used was equipped with wide-band-pass filters instead of the 490- and 450-nm narrow-band-pass filters generally used for pH measurements (27, 36, 39), the following results show that our determinations are valid. (i) Fluorescence intensity varied linearly with probe concentration (André et al., in press). (ii) Autofluorescence did not hamper our measurements since unlabeled cells yielded negligible signal. (iii) Data were very accurate since for each pH value, the coefficient of variation of the blue/purple intensity ratio did not exceed 5 to 10% (Fig. 4). (iv) Finally, after monensin was added to living cells, the pH of endocytic compartments led to that of extracellular medium (see below). The only problem which may occur with the use of wide-band-pass filters is that the intensity ratio may display smaller changes when the pH is varied. However, when the pH was increased from 5 to 7, our intensity

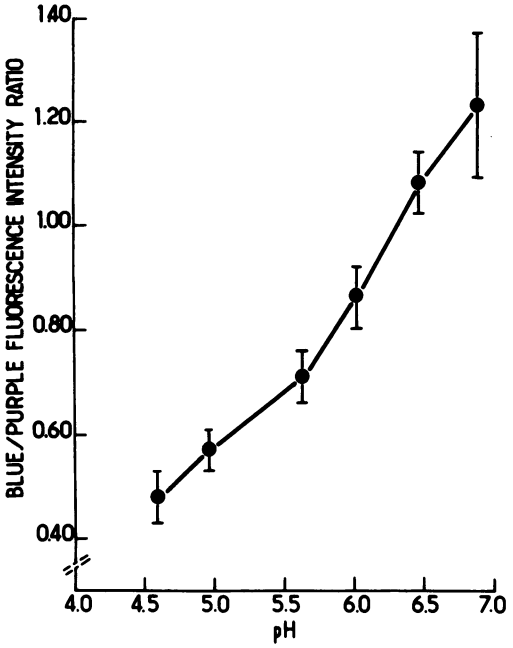


FIG. 4. pH calibration curve constructed with F-Dex-loaded and fixed macrophages. After incubation with F-Dex, uninfected macrophages were fixed with paraformaldehyde and then equilibrated with citrate-phosphate buffers of various pHs (4.61, 4.97, 5.63, 6.02, 6.47, and 6.89), each containing 20 μM monensin. For each pH, the blue/purple fluorescence intensity ratios of individual cells were determined. Each point on the graph represents the mean ± 1 SD of 12 to 23 cells.

ratio was increased by 2.19, which is very similar to the 2.58 value found by Horwitz and Maxfield (27) in studies made at the single-cell level with 450- and 490-nm narrow-band-pass filters.

**(ii) pH of late endocytic compartments.** To be sure that pH measurements of secondary lysosomes and PV were not biased by the presence of F-Dex in early endocytic compartments, cells were washed after F-Dex incubation and chased for various times at 34°C before fluorescence intensities were recorded. No change of the pH values could be observed whatever the chase time (Table 1).

In uninfected macrophages, the mean pH values of secondary lysosomes ranged between 5.17 and 5.48, which agree quite well with results obtained for other cell types (36). On the other hand, in infected macrophages, the mean

TABLE 1. Effect of chase time on the pH measurement of late endocytic compartments in uninfected macrophages

| Time of culture at 34°C (h) | Chase time after F-Dex incubation (min) | pH[a] |
|---|---|---|
| 24 | 0 | 5.42 ± 0.31 (6) |
| | 30 | 5.50 ± 0.28 (19)[b] |
| | 160 | 5.48 ± 0.21 (10)[b] |
| 48 | 0 | 5.34 ± 0.21 (8) |
| | 120 | 5.28 ± 0.21 (15)[b] |

[a] Mean ± 1 SD. Values in parentheses indicate the number of individual cells examined.

[b] pH measurements made after various chase times were not significantly different from those performed on unchased cells (Student and Fisher's *t* test).

TABLE 2. Comparative study of PV (infected macrophages) and secondary lysosomes (uninfected macrophages) pH values

| Expt | Time of culture at 34°C or after infection (h) | Monensin[a] | pH[b] | |
|---|---|---|---|---|
| | | | Uninfected macrophages | Infected macrophages |
| 1 | 24 | − | 5.48 ± 0.26 (35) | 5.26 ± 0.28 (30)[c] |
| | | + | >7.00 (10) | 6.64 ± 0.38 (25)[d] |
| | 48 | − | 5.30 ± 0.21 (23) | 4.82 ± 0.26 (15)[c] |
| | | + | 6.59 ± 0.27 (24) | 6.29 ± 0.39 (24)[d] |
| 2 | 24 | − | 5.17 ± 0.38 (18) | 4.74 ± 0.14 (18)[c] |
| | | + | 6.73 ± 0.21 (17) | 6.18 ± 0.33 (16)[d] |

[a] Fluorescence intensities were recorded in the presence (20 μM) or absence of monensin.

[b] Mean ± 1 SD. Values in parentheses indicate the number of individual cells examined.

[c] $P < 0.01$ compared with pH found for secondary lysosomes of uninfected macrophages (Student and Fisher's *t* test).

[d] $P < 0.01$ compared with pH found for secondary lysosomes of uninfected macrophages in the presence of monensin (Student and Fisher's *t* test).

pH of PV was slightly more acidic (by 0.2 to 0.5 pH units) and averaged 4.74 to 5.26 (Table 2). In each experiment, the difference between the pH of secondary lysosomes and that of PV was highly significant ($P < 0.01$). After monensin was added, the pH of both lysosomes and PV rapidly increased and reached stable, almost neutral values (Table 2). However, even after pH gradients were collapsed with monensin, PV exhibited a more acidic internal content than lysosomes, with the differences between the two sets of values being highly significant.

Digitization of video images also allowed examination of the fluorescence intensity of different areas of the same cell. At the magnification used, each pixel corresponded to an area of 1 $\mu m^2$. As an example, blue/purple ratios calculated from digitized images of cells excited with blue and purple wavelengths are presented in Fig. 5. Ratios are expressed in arbitrary units after the following conversion: $x = 8 + 2 \log_2 r$. It is clear that in both uninfected and infected macrophages, transformed ratios and thus pHs of the different areas were quite uniform. Furthermore, this analysis confirmed results obtained by measuring total cell fluorescence intensities and showed at the subcellular level that F-Dex-containing compartments are more acidic in infected (ratios of 5 to 6) than in uninfected (ratios of 6 to 7) macrophages.

## DISCUSSION

It has been previously suggested that ammonia production by *Leishmania* of the *mexicana* complex must increase the normally acidic phagolysosomal pH (14; Coombs and Alexander, cited in reference 9). Our results clearly demonstrated that this is not the case in our host cell-parasite combination. Although *L. amazonensis* amastigotes grew quite well in rat bone marrow-derived macrophages, PV were strongly acidified and constituted the main acidic compartment of infected cells. Most likely, these internal PV conditions are reached progressively and might be linked with the fusion of these organelles with secondary lysosomes as suggested by the experiments performed with the weak base DAMP. Thus, 6 h after infection, DAMP-stained secondary lysosomes were still detected and only some of the PV accumulated DAMP, whereas 24 to 48 h after infection, labeled lysosomes had almost completely disappeared but most if not all PV trapped DAMP. Depletion of secondary lysosomes has already been observed in *L. amazonensis*-infected

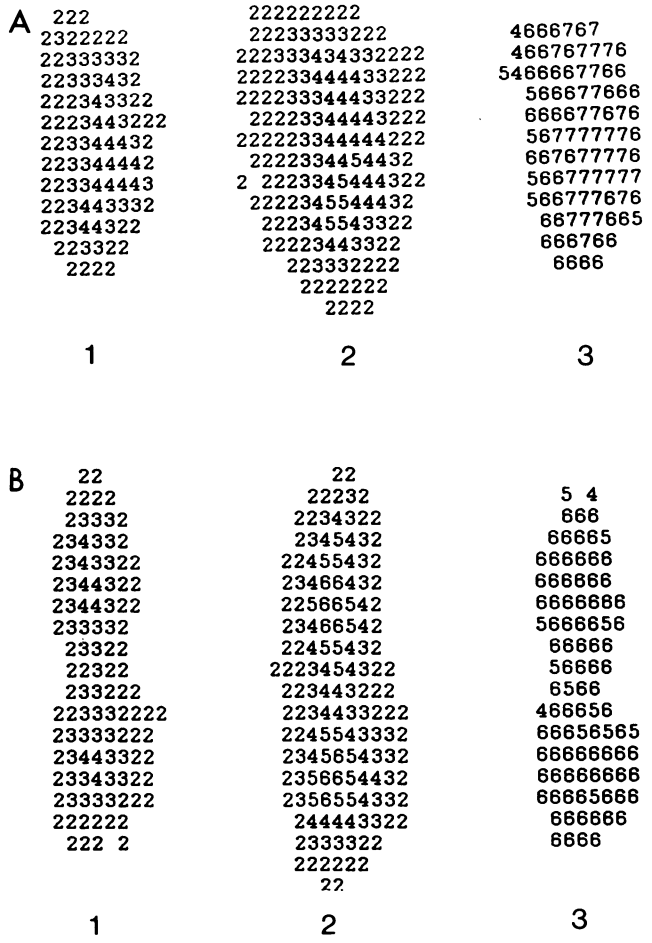


FIG. 5. Image digitization of F-Dex-loaded uninfected (A) and infected (B) macrophages. Cells were examined 24 h after the beginning of incubation at 34°C or after adding amastigotes. Digitized video images of cells excited with blue and purple wavelengths are shown in images 1 and 2, respectively. Each number of the digitized cells represents the fluorescence intensity of a 1-$\mu m^2$ area expressed in arbitrary units. For each 1-$\mu m^2$ area, the blue/purple fluorescence intensity ratio ($r$) was calculated. These ratios, transformed by the function $x = 8 + 2 \log_2 r$, are shown in image 3.

macrophages by using nondiffusible lysosomal markers (4, 6), indicating that DAMP labeling of PV is not merely due to a preferential accumulation of the weak base in PV to the detriment of lysosomes (20).

High fusion rates of lysosomes with PV might be of major importance for the formation of the huge PV which house *Leishmania* of the *mexicana* complex. However, how these vacuoles persist for several days in the cytoplasm of infected cells remains an intriguing question since, in the normal state, the lysosomal apparatus has been described as a rapidly intermixing organellar compartment (16, 46). According to this model, exchange between lysosomes would be mediated either by direct lysosome-lysosome fusion or through vesicular carriers, and maintenance of the integrity of the lysosomal compartment would require that these fusion processes be followed by fission events. Thus, an attractive hypothesis is that the presence of *Leishmania* parasites within the lysosomal compartment modifies its structural organization by impeding the fission events. Nevertheless, at least some of the lysosomal characteristics are preserved in PV since they exhibit an acidified content, and we have detected the presence of seven acid hydrolases in this compartment (4; Prina et al., in preparation).

Acidification of PV is very likely maintained by an active process partially ensured by the lysosomal proton-translocating ATPase inserted in the PV membrane. This idea is supported by the following results. (i) With the help of a quantitative assay, DAMP uptake by infected macrophages was reduced by more than 90% when incubation with the weak base took place at 4°C instead of 34°C (data not shown). (ii) The proton ionophore monensin inhibited the trapping of DAMP in infected cells by about 70% (data not shown) and rapidly increased the pH of PV by up to 1.5 units. However, we repeatedly found that the pH rise after monensin treatment was not as high and rapid for PV as for secondary lysosomes. This could be explained either by the apparently greater proton content of PV or by different membrane permeabilities of these organelles for monensin. A greater participation of a Donnan equilibrium in the acidification process of PV cannot be excluded either.

Unexpectedly, PV were found to be slightly more acidic than secondary lysosomes even though the volume of the former is greater by at least 1 order of magnitude (data not shown). A greater density of $H^+$ pumps in PV than in lysosomes or their different regulation in each type of organelle (C. C. Cain, D. M. Sipe, and R. F. Murphy, J. Cell Biol. **107**:808a, 1988) could explain these results. However, the possibility of an active participation of parasites in the acidification of PV must also be considered. Thus, a surface membrane proton-translocating ATPase, which probably extrudes protons to the outside and could be involved in the regulation of pH homeostasis (19, 48; D. Zilberstein and A. Gepstein, J. Cell. Biochem. Suppl. **13E**:127, 1989), was recently discovered in *Leishmania* species. Furthermore, release of dicarboxylic acids in the surrounding medium and concomitant decrease of extracellular pH were documented for *Leishmania* promastigotes (34).

Overall, these data completely agree with those obtained by Mukkada et al. (37) which suggested that *Leishmania* amastigotes are acidophilic organisms since several of their metabolic activities are optimum between pH 4 and 5.5. On the other hand, host lysosomal enzymes which are found in PV and whose activities are similar or greater in infected than in uninfected macrophage extracts are probably fully active in situ, and this could be connected to the presence of several *Leishmania* plasma membrane proteins resistant to proteolysis (12, 24, 28). The following hypothesis is thus emerging from these results. Amastigotes are not only resistant to the acidic and hydrolytic conditions of PV, but these special conditions are needed for long-term survival and growth of these parasites. Formation of PV by fusion of almost all secondary lysosomes would allow the access of large amounts of acid hydrolases to these compartments. Activities of these enzymes on exogenous substrates would provide amastigotes with nutrients such as amino acids and saccharides, whose transport into amastigotes would be ensured by the transmembrane pH gradient (19).

Most of the intracellular pathogens have developed strategies to escape the phagolysosomal environment. Thus, after the phagocytic process, *Shigella flexneri* (42), *Mycobacterium leprae* (15), *Rickettsia tsutsugamushi* (41), *Rickettsia typhi* (45), and *Trypanosoma cruzi* trypomastigotes (32, 38) reach the host cell cytoplasm where they multiply. On the contrary, some others such as *Legionella pneumophila* (27), *Nocardia asteroides* (8), *Listeria monocytogenes* (J. C. Wherry, P. H. Schlesinger, D. J. Krogstad, S. A. Moser, L. S. Mayorga, and P. D. Stahl, FASEB J. **3**:A319,

1989), *Mycobacterium microti*, *M. tuberculosis* (25), *M. avium* (17), and *Toxoplasmi gondii* (43) inhibit the acidification of phagosomes and/or the phagosome-lysosome fusion. Therefore, to our knowledge, *Leishmania* species share their capacity to multiply within at least partially functional phagolysosomes with only a few other microorganisms such as *Coxiella burnetii* (23) and *Mycobacterium lepraemurium* (25). This provides the impetus for further biological study of these parasites. It is also clear from these results that studies of pathogen-host cell interactions by using a cell biology approach must have important repercussions in the applied fields of drug development and targeting, which must take into account the properties and accessibility of the compartments where parasites reside. The same is true for vaccine development since it is becoming increasingly clear that antigen processing and presentation could vary according to their intracellular localization (18, 29, 33). Therefore, knowledge about the functional state of the organelles which house pathogens could be of paramount importance for the design of vaccine molecules.

## ACKNOWLEDGMENTS

This work was supported by the Institut Pasteur and the Centre National de la Recherche Scientifique (UA 04 1113). E. Prina is a recipient of a Fondation Marcel Mérieux student fellowship.

We are grateful to Dana Baran for the careful reading of the manuscript, to Danielle Antoine for the graphic work, and to Chantal Maczuka for typing the manuscript.

## LITERATURE CITED

1. **Anderson, R. G. W., J. R. Falck, J. L. Goldstein, and M. S. Brown.** 1984. Visualization of acidic organelles in intact cells by electron microscopy. Proc. Natl. Acad. Sci. USA **81**:4838–4842.
2. **Anderson, R. G. W., and L. Orci.** 1988. A view of acidic intracellular compartments. J. Cell Biol. **106**:539–543.
3. **Antoine, J.-C., C. Jouanne, A. Ryter, and J.-C. Benichou.** 1988. *Leishmania amazonensis*: acidic organelles in amastigotes. Exp. Parasitol. **67**:287–300.
4. **Antoine, J.-C., C. Jouanne, A. Ryter, and V. Zilberfarb.** 1987. *Leishmania mexicana*: a cytochemical and quantitative study of lysosomal enzymes in infected rat bone marrow-derived macrophages. Exp. Parasitol. **64**:485–498.
5. **Avrameas, S., and T. Ternynck.** 1971. Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry **8**:1175–1179.
6. **Barbieri, C. L., K. Brown, and M. Rabinovitch.** 1985. Depletion of secondary lysosomes in mouse macrophages infected with *Leishmania mexicana amazonensis*. Z. Parasitenkd. **71**:159–168.
7. **Barrett, A. J., and M. F. Heath.** 1977. Lysosomal enzymes, p. 19–145. *In* J. T. Dingle (ed.), Lysosomes: a laboratory handbook, 2nd ed. Elsevier/North-Holland Biomedical Press, Amsterdam.
8. **Black, C. M., M. Palieschekey, B. L. Beaman, R. M. Donovan, and E. Goldstein.** 1986. Acidification of phagosomes in murine macrophages: blockage by *Nocardia asteroides*. J. Infect. Dis. **154**:952–958.
9. **Bray, R. S., and J. Alexander.** 1987. *Leishmania* and the macrophage, p. 211–233. *In* W. Peters and R. Killick-Kendrick (ed.), The leishmaniases in biology and medicine I. Academic Press, Inc. (London), Ltd., London.
10. **Chang, K.-P.** 1980. Human cutaneous *Leishmania* in a mouse macrophage line: propagation and isolation of intracellular parasites. Science **209**:1240–1242.
11. **Chang, K.-P.** 1980. Endocytosis of *Leishmania*-infected macrophages. Fluorometry of pinocytic rate, lysosome-phagosome fusion and intralysosomal pH, p. 231–234. *In* H. Van den Bossche (ed.), The host invader interplay. Elsevier/North-Holland Biomedical Press, Amsterdam.
12. **Chang, K.-P.** 1983. Cellular and molecular mechanisms of intracellular symbiosis in leishmaniasis. Int. Rev. Cytol. **14** (suppl.):267–305.
13. **Collot, M., D. Louvard, and S. J. Singer.** 1984. Lysosomes are associated with microtubules and not with intermediate filaments in cultured fibroblasts. Proc. Natl. Acad. Sci. USA **81**:788–792.
14. **Coombs, G. H.** 1982. Proteinases of *Leishmania mexicana* and other flagellate protozoa. Parasitology **84**:149–155.
15. **Evans, M. J., and L. Levy.** 1972. Ultrastructural changes in cells of the mouse footpad infected with *Mycobacterium leprae*. Infect. Immun. **3**:238–247.
16. **Ferris, A. L., J. C. Brown, R. D. Park, and B. Storrie.** 1987. Chinese hamster ovary cell lysosomes rapidly exchange contents. J. Cell Biol. **105**:2703–2712.
17. **Frehel, C., C. de Chastellier, T. Lang, and N. Rastogi.** 1986. Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic *Mycobacterium avium*. Infect. Immun. **52**:252–262.
18. **Germain, R. N.** 1986. The ins and outs of antigen processing and presentation. Nature (London) **322**:687–689.
19. **Glaser, T. A., J. E. Baatz, G. E. Kreishman, and A. J. Mukkada.** 1988. pH homeostasis in *Leishmania donovani* amastigotes and promastigotes. Proc. Natl. Acad. Sci. USA **85**:7602–7606.
20. **Goren, M. B., C. L. Swendsen, J. Fiscus, and C. Miranti.** 1984. Fluorescent markers for studying phagosome-lysosome fusion. J. Leukocyte Biol. **36**:273–292.
21. **Graham, R. C., and M. J. Karnovsky.** 1966. The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. **14**:291–302.
22. **Graham, R. C., U. Lundholm, and M. J. Karnovsky.** 1965. Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. J. Histochem. Cytochem. **13**:150–152.
23. **Hackstadt, T., and J. C. Williams.** 1981. Biochemical stratagem for obligate parasitism of eucaryotic cells by *Coxiella burnetii*. Proc. Natl. Acad. Sci. USA **78**:3240–3244.
24. **Handman, E., G. F. Mitchell, and J. W. Goding.** 1981. Identification and characterization of protein antigens of *Leishmania tropica* isolates. J. Immunol. **126**:508–512.
25. **Hart, P. D., J. A. Armstrong, C. A. Brown, and P. Draper.** 1972. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infect. Immun. **5**:803–807.
26. **Helenius, A., I. Mellman, D. Wall, and A. Hubbard.** 1983. Endosomes. Trends Biochem. Sci. **8**:245–250.
27. **Horwitz, M. A., and F. R. Maxfield.** 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. J. Cell Biol. **99**:1936–1943.
28. **Kahl, L. P., and D. McMahon-Pratt.** 1987. Structural and antigenic characterization of a species- and promastigote-specific *Leishmania mexicana amazonensis* membrane protein. J. Immunol. **138**:1587–1595.
29. **Kaye, P. M.** 1987. Antigen presentation and the response to parasitic infection. Parasitol. Today **3**:293–299.
30. **Klebanoff, S. J.** 1980. Oxygen intermediates and the microbicidal event, p. 1105–1137. *In* R. van Furth (ed.), Mononuclear phagocytes. Functional aspects II. Martinus Nijhoff Publishers, The Hague, The Netherlands.
31. **Klebanoff, S. J., R. M. Locksley, E. C. Jong, and H. Rosen.** 1983. Oxidative response of phagocytes to parasite invasion. CIBA Found. Symp. **99**:92–112.
32. **Kress, Y., B. R. Bloom, M. Witter, A. Rowen, and H. T. Tanowitz.** 1975. Resistance of *Trypanosoma cruzi* to killing by macrophages. Nature (London) **257**:394–396.
33. **Long, E. O., and S. Jacobson.** 1989. Pathways of viral antigen processing and presentation to CTL: defined by the mode of virus entry? Immunol. Today **10**:45–48.
34. **Marr, J. J.** 1980. Carbohydrate metabolism in *Leishmania*, p. 313–340. *In* M. Levandowsky and S. H. Hutner (ed.), Biochemistry and physiology of protozoa, vol. 3. Academic Press, Inc., Orlando, Fla.
35. **Matteoni, R., and T. E. Kreis.** 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. J. Cell Biol. **105**:1253–1265.

36. **Maxfield, F. R.** 1985. Acidification of endocytic vesicles and lysosomes, p. 235–257. *In* I. Pastan and M. C. Willingham (ed.), Endocytosis. Plenum Publishing Corp., New York.
37. **Mukkada, A. J., J. C. Meade, T. A. Glaser, and P. Bonventre.** 1985. Enhanced metabolism of *Leishmania donovani* amastigotes at acid pH: an adaptation for intracellular growth. Science **229**:1099–1101.
38. **Nogueira, N., and Z. A. Cohn.** 1976. *Trypanosoma cruzi*: mechanism of entry and intracellular fate in mammalian cells. J. Exp. Med. **143**:1402–1420.
39. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75**:3327–3331.
40. **Rabinovitch, M.** 1985. The endocytic system of *Leishmania*-infected macrophages, p. 611–619. *In* R. van Furth (ed.), Mononuclear phagocytes. Characteristics, physiology and function. Martinus Nijhoff, Dordrecht, The Netherlands.
41. **Rikihisa, Y., and S. Ito.** 1979. Intracellular localization of *Rickettsia tsutsugamushi* in polymorphonuclear leukocytes. J. Exp. Med. **150**:703–708.
42. **Sansonetti, P. J., A. Ryter, P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51**:461–469.
43. **Sibley, L. D., E. Weidner, and J. L. Krahenbuhl.** 1985. Phagosome acidification blocked by intracellular *Toxoplasma gondii*. Nature (London) **315**:416–419.
44. **Ternynck, T., and S. Avrameas.** 1972. Polyacrylamide-protein immunoadsorbents prepared with glutaraldehyde. FEBS Lett. **23**:24–28.
45. **Walker, T. S., and H. H. Winkler.** 1981. Interactions between *Rickettsia prowazekii* and rabbit polymorphonuclear leukocytes: rickettsiacidal and leukotoxic activities. Infect. Immun. **31**:289–296.
46. **Weisman, L. S., and W. Wickner.** 1988. Intervacuole exchange in the yeast zygote: a new pathway in organelle communication. Science **241**:589–591.
47. **Yamashiro, D. J., and F. R. Maxfield.** 1987. Acidification of morphologically distinct endosomes in mutant and wild-type Chinese hamster ovary cells. J. Cell Biol. **105**:2723–2733.
48. **Zilberstein, D., and D. M. Dwyer.** 1988. Identification of a surface membrane proton-translocating ATPase in promastigotes of the parasitic protozoan *Leishmania donovani*. Biochem. J. **256**:13–21.