RECEPTOR-MEDIATED ENTRY OF β-GLUCURONIDASE INTO THE PARASITOPHOROUS VACUOLES OF MACROPHAGES INFECTED WITH LEISHMANIA MEXICANA AMAZONENSIS*

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Leishmania are protozoa that infect humans and certain other mammals in tropical and subtropical countries. Different species and subspecies of the parasite induce a wide spectrum of cutaneous, mucocutaneous, or visceral disease. The nonflagellated form of the parasite, the amastigote, is essentially an obligatory parasite of mononuclear phagocytes (1, 2). Infected macrophages can survive for at least several weeks in culture and the infection is quite compatible with replication of the host cells (3). Leishmania amastigotes lodge and multiply within parasitophorous vacuoles (p.v.), assumed to be modified phagolysosomes. This assumption is supported by experiments in which the secondary lysosomes of macrophages were loaded with electron-dense tracers such as thorium dioxide or saccharate iron oxide before infection of the cultures with Leishmania. Transmission electron microscopy revealed the presence of the colloids within the p.v. and images suggesting fusion of the labeled secondary lysosomes with the vacuoles were also documented (4–6). However, only rarely was electron-dense acid phosphatase product detected in the lumen of the p.v. (4, 5, 7).

Macrophages infected with Leishmania thus provide an interesting system to determine how intracellular parasites may affect the numbers, function, synthesis, assembly, and recirculation of the vacuolar and lysosomal components of the host cell. In the present study we show that radioiodinated rat preputial gland β-glucuronidase, a ligand recognized and internalized via macrophage surface receptors specific for mannose-terminated ligands (8, 9), can enter the parasitophorous vacuoles when given to macrophage cultures either before or after infection with L. mexicana amazonensis. We chose this species of Leishmania because of the large size of the vacuoles it induces in the host cells.

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Abbreviations used in this paper: DME, Dulbecco's modified Eagle's minimum essential medium; FBS, fetal bovine serum; HS, horse serum; LCM, L cell-conditioned medium; man-BSA, mannose-bovine serum albumin; p.v., parasitophorous vacuoles; PBS, phosphate-buffered, Ca-, Mg-free saline; TCA, trichloroacetic acid.
Materials and Methods

Animals. Adult golden hamsters were bred at and provided by the Division of Parasitology, New York University School of Medicine. Female Swiss-Webster mice, 20–25 g body weight were purchased from Taconic Farms, Inc., Germantown, NY.

Media. Ca ++, Mg ++-free phosphate-buffered saline (PBS) contained 5 mm potassium phosphate buffer and 138 mM NaCl and was adjusted to pH 7.2. Dulbecco’s modified Eagle’s minimal essential medium (DME), horse serum (HS), and fetal bovine serum (FBS) were obtained from Gibco Laboratories, Grand Island, NY. Conditioned medium (LCM) was collected from confluent L cell fibroblast cultures incubated for 7 d in serum-free medium (10).

Preparation of the Ligands. β-glucuronidase was purified to homogeneity from rat preputial glands (11). Mannose-bovine serum albumin (Man-BSA) was prepared as described elsewhere (12) and contained 20–40 mol of sugar per ml of protein. Ligands were iodinated by the chloramine T method (13) to a specific activity of 1–10 μCi/μg.

Leishmania Strain and Preparation of the Amastigote Inoculum. L. mexicana amazonesis LV 79, originally obtained from the Dept. of Parasitology, Liverpool School of Tropical Medicine, England, was kindly provided by Dr. J. P. Dedet (Institut Pasteur, Paris). Amastigote suspensions were injected subcutaneously in the paws of hamsters, and 4–6 wk later the granulomas were excised and minced. Tissue debris was removed by slow centrifugation (40 g, 10 min), and the parasites were recovered and washed by three centrifugation cycles at 1,000 g for 10 min (10).

Macrophage Cultures. Bone marrow-derived macrophages were obtained by explanting 1.5 × 10⁵ mouse bone marrow cells into each of 16-mm diam wells of Cluster plates (Costar, Data Packaging, Cambridge, MA) containing 0.5 ml of DME enriched with 10% HS, 10% of LCM, 50 U of penicillin, and 50 μg/ml of streptomycin. The cultures were used on the 5th d, at which time they contained between 3 × 10⁵ and 5 × 10⁵ macrophages. For light microscopic radioautography, cells were grown on 13-mm glass coverslips placed in the wells. For electron microscopic radioautography, cells were grown in 35-mm dishes, and for radiometric determinations of ligand uptake and degradation, macrophages were grown directly on tissue culture plastic in cluster plates. Macrophages were grown at 37°C in a humidified 5% CO₂-air atmosphere.

Infection of the Macrophage Cultures. The parasites were suspended in DME containing 10% FBS and 2.5% LCM, and the cultures were infected with an estimated multiplicity of two to three amastigotes per macrophage. Since the strain of Leishmania used is temperature sensitive (14), infected cultures were transferred to an incubator adjusted to a temperature of 34°C. The percent of infection varied between 50 and 80% in different experiments.

Uptake Studies. For time course studies, 125I-β-glucuronidase was added to each well containing 0.4 ml DME, with 10% S, 2.5% LCM, and 10 mM Hepes. Ligand was added at a concentration of 20 μg/ml (containing cold carrier) and a specific activity of 3–4 × 10⁶ cpm/μg. To determine nonspecific uptake, companion wells received 1 mg/ml mannan. For studies using 125I-Man-BSA as the ligand, Man-BSA was added at a concentration of 1 μg/ml (with added cold) and ~10⁶ cpm/μg. At various times after addition of the ligand, the medium was removed (for degradation studies) and the cells were washed twice with PBS. The cell monolayers were solubilized in 0.1% Triton X-100 and counted by means of a gamma counter. Ligand degradation (using Man-BSA) was measured by determining the radioactivity liberated from Man-BSA in the culture medium after precipitation with trichloroacetic acid (TCA) (15).

Radioautography. For radioautography experiments, ligand additions were as follows: β-glucuronidase was added at 20 μg/ml and a specific activity of ~2.5 × 10⁶ cpm/μg (2 × 10⁵ cpm/well); Man-BSA was added at 1 μg/ml and ~6 × 10⁵ cpm (2 × 10⁵ cpm/well). For time course studies, macrophage cultures (1–2 d postinfection) were incubated with labeled ligands for periods of time between 30 min and 50 h. Uninfected macrophages were similarly treated. In pulse-chase experiments, infected and noninfected macrophages were incubated with ligand for 18 h, washed, and incubated in ligand-free medium for varying periods of time before fixation. In “pre-load” experiments, cultures were incubated with 125I-β-glucuronidase, washed, infected, and maintained in ligand-free medium until fixation.

For light microscopic radioautography, after incubation with ligand the macrophages on coverslips were washed three times with PBS and fixed for 30 min at room temperature with
either 9:1 ethanol/acetic acid or 2% glutaraldehyde in PBS. After fixation the cultures were
washed in distilled water, dried, and the coverslips were attached to microscope slides by means
of 'Permunt' (Fisher Scientific Co., Pittsburgh, PA). The slides were coated with NTB-2
nuclear track emulsion (Eastman Kodak Co., Rochester, NY) (16). After an exposure of 2-4 d,
the emulsion was developed in D19, fixed, and the cells were lightly counterstained with Diff
Quick (American Scientific Products Div., American Hospital Supply Corp., McGraw Park,
IL).

For electron microscopic radioautography, macrophages cultivated on 35-mm dishes after
incubation with the ligand were fixed in glutaraldehyde, postfixed in 1% osmium tetroxide,
stained with magnesium uranyl acetate, and embedded in Epon 812 (Ernest F. Fullam, Inc.,
Schenectady, NY). Representative areas were cut out and re-embedded. Pale gold sections were
prepared and coated with Ilford L4 emulsion (Polysciences, Inc., Warrington, PA) as described
elsewhere (17). After a 2-wk exposure the emulsion was developed with D19 and fixed. The
sections were picked up on 200 mesh grids, stained with uranyl acetate and lead citrate, and
viewed with a JEOL 100 S electron microscope.

Results

Light Microscopic Radioautography. Marrow-derived macrophage cultures were estab-
lished on coverslips and infected with Leishmania. 1 or 2 d after infection, 125I-β-
glucuronidase was added to the cultures. At various intervals between 30 min and 50
h, the monolayers were fixed and processed for light microscopic radioautography.
To determine whether the uptake was mediated by the mannose receptor, some
cultures were incubated with the ligand in the presence of 1 mg/ml mannan.

Radioautographs of infected macrophages were scored as positive when the grain
density over the p.v. was detectably higher than the grain density over both the
remainder of the cell and the intercellular background. This criterion could be met
within a large range of grain densities overlying the vacuoles. In the usual prepara-
tions, 5-10 grains over a medium sized vacuole (80-100 μm²) defined a lightly but
definitely labeled infected cell. Positive vacuoles were rarely found before 4 h of
incubation with the ligand. Consistent labeling was observed at 4 h and at later time
periods. Fig. 1 summarizes the results of three separate experiments and shows that
the percent of macrophages bearing at least one positive vacuole increased with the
incubation time, from ~15% at 4 h to ~80% at 50 h. At each time period grain
densities varied markedly between different vacuoles, but as the incubation time
increased, the proportion of heavily labeled vacuoles (>50 grains per 100 μm²)
increased. Figs. 2a and b provide examples of positive radioautographs over the p.v.

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![Fig. 1. Light microscopic radioautography of macrophages infected with L. mexicana amazonensis. Percent of macrophages with labeled parasitophorous vacuoles as a function of the length of incubation of the cultures with 125I-β-glucuronidase. The curves summarize the results of three separate experiments. Each point represents the average of 2-4 coverslips, with at least 50 macrophages scored per coverslip.](image)
Fig. 2. Light microscopic radioautographs of macrophages incubated with radiolabeled β-glucuronidase. The length of the bar represents 10 μm. a, b, and c show macrophages fixed 20 h after continuous incubation with the ligand. (a) Microscope field with six macrophages displaying labeled p.v. Arrowhead points to Leishmania amastigote. Note the scarcity of grains over the non-p.v. cytoplasm. (b) Well-infected macrophage with high grain density over p.v. and a clearly but less labeled cytoplasm. (c) Macrophages incubated with the ligand for 20 h in the presence of 1 mg/ml mannan. Very few grains are seen over the cells. (d) Macrophages preloaded with the radiolabeled ligand for 5 h, washed, infected, and cultivated for 48 h without any added ligand. Moderate but definite labeling of the p.v.
of macrophages incubated with the ligand for 20 h. In most instances (Fig. 2a) infected cells had few grains over the cytoplasm (besides the grains over the p.v.). In only a few cells (e.g., Fig. 2b) was the non-p.v. cytoplasm moderately labeled. Furthermore, addition of mannan with the ligand (Fig. 2c) markedly reduced both the percent of labeled macrophages (e.g., from 80% to 15%, averages of triplicates) and the grain density over the positive vacuoles.

The results indicate that an exogenous ligand accumulates and persists within the parasitophorous vacuoles, but they do not demonstrate the means of delivery of the ligand. The ligand could be transferred to p.v. by fusion of prelysosomal vesicles, of secondary lysosomes with the vacuoles, or by a combination of these mechanisms. To determine if ligand could be transferred to vacuoles from preformed lysosomes, the following preloading experiment was performed. Noninfected macrophages were incubated with $^{125}$I-$\beta$-glucuronidase for 5 or 18 h, washed, infected, and cultivated for an additional period of 48 h in the absence of added ligand. In a typical experiment, after a loading period of 18 h, 54% of the infected macrophages displayed labeled parasitophorous vacuoles (average of three coverslips). Fig. 2d illustrates positive p.v. radioautographs in macrophages preloaded with the ligand for 5 h, washed, infected, and fixed after an additional culture period of 48 h. Since the bulk of the internalized ligand should have been delivered to the lysosomal compartment during the preloading period, this result indicates that secondary lysosomes can fuse with and deliver ligand to the p.v. The possibility that prelysosomal vesicles also carried ligand to the vacuoles cannot be excluded but is improbable in view of the prolonged incubation of cells with ligand that was required for detectable labeling of the vacuoles in time course experiments (Fig. 1).

**Pulse-chase Experiments.** To evaluate the persistence of the label in p.v., infected macrophages were pulsed with ligand for 24 h, washed, incubated in ligand-free medium, and fixed at varying periods thereafter. In one such experiment, the percent of macrophages with positive p.v. after postincubation for 8, 24, or 48 h was respectively 85, 83, and 39 (averages of triplicates). Grain density over the vacuoles was distinctly reduced in the 48-h group. Thus, some ligand appeared to be associated with vacuoles for at least 48 h after a 24-h period of exposure to the ligand. This result agrees with the known stability of internalized $\beta$-glucuronidase (13).

**Electron Microscopic Radioautography.** The light microscopic observations suggested but did not prove that ligand was taken up into the p.v. Indeed, the grains could have arisen from label distributed around but not within the vacuoles. The true vacuolar location of the ligand was confirmed by means of radioautography at the electron microscopic level. Control and infected macrophages were incubated with $^{125}$I-$\beta$-glucuronidase for 20 h, washed, fixed, and embedded in Epon, and thin sections were processed for electron-microscope radioautography. Fig. 3 shows a radioautograph of a noninfected macrophage. The cell, which was fixed after 20 h of incubation with the ligand, displays numerous silver grains mostly associated with secondary lysosomes. Fig. 4 shows a low magnification picture of a radioautograph of an infected macrophage similarly incubated with the radiolabeled ligand; most of the grains are associated with the p.v. Two pictures of radioautographs of infected macrophages, taken at a higher magnification, are shown in Figs. 5a and b. Again, silver grains are predominantly associated with the p.v. While a few grains are present over the remaining cytoplasm, they are not clearly associated with secondary lysosomes.
Fig. 3. Electron microscopic radioautograph of noninfected macrophage incubated with radiolabeled β-glucuronidase for 20 h before fixation. Silver grains are mainly associated with dense secondary lysosomes. Bar indicates 1 μm.
Studies with $^{125}$I-Man-BSA. Experiments similar to those described above were performed with infected macrophages incubated with $^{125}$I-man-BSA, a ligand rapidly degraded after uptake via the mannose receptor (13). In no instance were p.v. distinctly labeled after incubation of macrophages with the iodinated man-BSA. Together with the uptake studies summarized below, these experiments show that man-BSA, in contrast with $\beta$-glucuronidase, is rapidly degraded within the lysosomal compartment and this probably precludes delivery of the ligand to the p.v.

Uptake Studies. Infected and noninfected macrophage cultures were incubated with iodinated $\beta$-glucuronidase or man-BSA. At different time periods cell-associated counts (for both ligands) or TCA-soluble counts (for man-BSA) were determined. Counts are expressed as percentage of the initial input after correction for mannan controls. In the experimental results summarized in Fig. 6, it can be seen that the
Fig. 5. Electron microscopic radioautographs of infected macrophages, from the same preparation as Fig. 4. Bar represents 1 μm. In both a and b most grains are found over p.v. The few grains over the cytoplasm are not clearly associated with secondary lysosomes. A tangentially cut Leishmania is indicated by arrow in b.
uptake of β-glucuronidase increased as the incubation period progressed. In contrast, man-BSA was rapidly degraded, with a time course of uptake showing increasing accumulation of TCA-soluble counts in the medium (Fig. 7). In each case the time course of uptake was similar in infected and noninfected cells.

Discussion

We have demonstrated that β-glucuronidase is taken up by macrophages infected with *Leishmania* and penetrates the parasitophorous vacuoles. Since this conclusion rests on the distribution of grains in light and electron microscopic radioautographs, the proportion of the radioactivity in the p.v. associated with intact as opposed to partially degraded enzyme molecules is not yet known. The semi-quantitative radioautographic observations of the persistence of the label in many vacuoles 24–48 h after removal of the ligand from the culture medium certainly supports the relatively long intracellular life of the enzyme in the present experiments.
The fate of the mannose receptor-ligand complex in macrophages has been studied in detail (13, 18). Once bound to the cell surface receptor, ligand is internalized (at 37°C) and delivered to lysosomes, while the receptor recycles back to the cell surface. Apparently, receptor-ligand complexes move into an acidic, presumably prelysosomal vesicle, where dissociation of the complex occurs, although the actual mechanism involved in these steps is unknown. After receptor-ligand dissociation, the ligand could be transferred to Leishmania-containing vacuoles by fusion with prelysosomal vesicles or of secondary lysosomes. The fact that vacuolar labeling was also observed in experiments where the cells were first loaded with β-glucuronidase and then infected suggests that the ligand had been delivered to secondary lysosomes and was then transferred through fusion to the p.v. This result agrees with previous observations using electron-opaque tracers (4-6). Although the present data support transfer from secondary lysosomes, delivery by means of prelysosomal ligand-containing vesicles cannot be excluded.

An interesting observation relates to the scarcity of grains over the cytoplasm of infected cells that carried labeled p.v. (Figs. 2, 4, and 5). In these and in previous ultrastructural studies (7), fewer lysosomes were also seen in the cytoplasm of infected cells. In addition, a clear reduction in the number of acid phosphatase or aryl-sulfatase positive granules was seen in the cytoplasm of Leishmania-infected peritoneal macrophages (C. B. Mestriner and M. Rabinovitch, manuscript in preparation). Thus, the reduction in the cytoplasmic label in infected macrophages may be related to the diminished number of lysosomes in the infected cell. It remains to be seen whether this apparent reduction in lysosome numbers is related to lysosome fusion with the p.v. and/or with the plasma membrane. Another possibility is that the reduction follows the inhibition of the synthesis or assembly of lysosomal components in infected cells.

Finally, in vivo studies have shown that β-glucuronidase is rapidly cleared from the circulation by Kupffer cells of the liver (19). Since this ligand accumulates within the p.v., it may be possible to devise mannosylated compounds able to target leishmanicidal drugs to the p.v. of infected macrophages.

Summary

125I-labeled rat preputial gland β-glucuronidase was shown by light and electron microscopic radioautography to accumulate within the parasitophorous vacuoles of in vitro derived bone marrow macrophages infected with Leishmania mexicana amazonensis. β-glucuronidase uptake was mediated by the mannose receptor, since the penetration of the ligand was inhibited by mannan. Uptake was detected as soon as 4 h after incubation of infected cells with the ligand, and increased at 24 and 48 h. The label persisted in the vacuoles for at least 24 h after a 24-h pulse with the ligand, a finding compatible with the relatively long half-life of labeled β-glucuronidase in normal macrophages. Parasitophorous vacuoles were also labeled in macrophages exposed to the ligand only before infection, indicating that secondary lysosomes containing the ligand fused with the parasitophorous vacuoles. Another mannosylated ligand, mannose-BSA, which, in contrast to β-glucuronidase, is rapidly degraded in macrophage lysosomes, did not detectably accumulate in the vacuoles. The results support and extend information previously obtained with electron opaque tracers that emphasizes the phagolysosomal nature of Leishmania parasitophorous vacuoles. In
addition, the results suggest that appropriate mannosylated molecules may be used as carriers for targeting of leishmanicidal drugs to the parasitophorous vacuoles of infected macrophages.

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