Transfer of Zymosan (Yeast Cell Walls) to the Parasitophorous Vacuoles of Macrophages Infected with *Leishmania amazonensis*

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Summary

Leishmania are flagellated protozoan parasites which, in their amastigote stages, survive and multiply within phagolysosome-like parasitophorous vacuoles (PV) of mammalian macrophages (MØ). This study develops an earlier ultrastructural, incidental observation that zymosan particles (Z) were transferred to the PV of macrophages infected with *Leishmania amazonensis*. In the present report, a pulse-chase light microscopic assay was used to delineate several features of the Z transfer. The assay reflects both the movement of internalized particles to a position adjoining a PV, and their delivery to the vacuoles. Transfer was selective, in the sense that Z, β-glucan or heat-killed yeast particles were transferred, whereas latex beads, aldehyde-fixed, or immunoglobulin G-coated erythrocytes were not. This selectivitly may be related to the high density of carbohydrate ligands displayed on the surface of yeast-derived particles, to ligand resistance to lysosomal degradation or to signals encoded in the cytosolic tails of the receptors involved in particle recognition. A few Z particles could be found within PV after 1 h of incubation with infected MØ, but chase periods of several hours at 34°C were required for particle transfer to the PV in a substantial proportion of the MØ. Ammonium chloride, chloroquine, or monensin, compounds which increase the pH in acidified compartments, substantially enhanced the transfer of Z particles. Finally, transfer was inhibited by cytochalasin D, but was unaffected by the antitubulin nocodazole. Although it is not yet known if particle transfer occurs by fusion of donor vesicles with PV or by interiorization of the former into the latter, the model described should be useful in the study of the interactions between large phagocytic vesicles and the modulation of those interactions by cellular, parasitic, and environmental signals.

Leishmania are unicellular eukaryotic parasites, which in their incompletely flagellated amastigote stage, lodge and multiply within membrane-bound parasitophorous vacuoles (PV)[1] of mammalian macrophages (MØ) (1, 2). These vacuoles are assumed to be phagolysosomes because they are acidified (3), contain lysosomal enzymes (4), and acquire, presumably by fusion with lysosomes or other endocytic vesicles, electron opaque colloids (5) or molecular ligands taken up by receptor-mediated endocytosis (6, 7). In addition, lysosomal glycoproteins (lgp) 110 and 120 (8) and in cryosections, the cation-independent mannose-6 phosphate receptor, a prelysosomal marker (D. G. Russell, personal communication), have been detected in the PV membranes of *Leishmania amazonensis*-infected MØ. Most of these studies have been performed with *Leishmania* of the *mexicana* complex, which typically induce the formation of large and rather stable PV, which contain numerous amastigotes.

In the course of experiments in which yeast cell walls (zymosan, Z) were used in induce lysosomal enzyme secretion by *L. amazonesis*-infected MØ, transmission electron microscopy observations revealed the presence of Z particles within the PV of the phagocytes (9). This result contrasted with earlier findings that other particulates such as latex beads or glutaraldehyde-fixed red blood cells did not enter PV (10). Both *Leishmania* amastigotes and Z particles are quite stable within host cells, and are easily identified by light and electron microscopy. Furthermore, at low parasite loads the infection is well tolerated by the host cells, as shown by cinematographic observations of dividing infected marrow phagocytes (11). Thus, the vesicular and quantal transfer of the particles to the PV of infected MØ offers a useful tool for the study of cellular and biochemical mechanisms which underlie the interactions between large phagocytic vesicles.

In the present report a pulse-chase light microscopic assay

[1] *Abbreviations used in this paper:* CQ, chloroquine; GRC, glutaraldehyde-fixed red blood cells; MØ, macrophage(s); PV, parasitophorous vacuole(s); Z, zymosan.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/09/0639/08 $2.00
Volume 176 September 1992 639–646

has been used to examine certain features and requirements of the transfer of particles to the PV of *L. amazonensis*-infected MØ.

Materials and Methods

Media. Ca^{2+} and Mg^{2+}-free PBS, pH 7.3, contained 138 mM NaCl and 6.7 mM potassium phosphate buffer. HBSS and FCS were from Flow Laboratories, Inc. (McLean, VA), and RPMI 1640 was from Gibco Laboratories (Grand Island, NY). HBSS was supplemented with 25 mM Mops [3-(*N*-morpholino)propane sulfonate] ("HBSS-Mops"). For MØ cultures, RPMI was supplemented with 2 g/l sodium bicarbonate, 10% FCS, 20 mM Hepes, and 20 μg/ml gentamycin (complete medium).

Chemicals. Nocodazole (methyl[5-(2-thienylcarbonyl)-1*H*-benzimidazol-2-yl]carbamate), monensin and cytochalasin D were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of nocodazole (at 1 mM) and of cytochalasin D (at 4 mg/ml) were prepared in dimethylsulfoxide. Monensin was dissolved at 5 mM in ethanol. All stock solutions were stored at −20°C.

Particles. Dehydrated baker's yeast (*Saccharomyces cerevisiae*) and zymosan A were from Sigma Chemical Co. Polystyrene carboxylated beads (2.02 μm) were from Dow Diagnostics (Indianapolis, IN), and polystyrene noncarboxylated beads (1.06 and 3.0 μm) from Sigma Chemical Co. Beads were washed twice (10,000 g, 10 min), resuspended in 5% glucose, and added to the culture medium. Yeast particles were suspended in PBS, autoclaved, washed four times with sterile PBS, resuspended to 1.5 × 10^8 particles/ml, and stored at −20°C. β-glucan particles were prepared from baker's yeast which was autoclaved for 45 min in 1.5 N NaOH, washed, successively extracted with water, 3% acetic acid, water, ethanol, acetone, and dried (12). Glutaraldehyde-fixed sheep and mouse red blood cells (GRC) were prepared as described (13).

Animals. Male BALB/c mice, 20–25 g body wt, provided by the Animal Facility of the Pasteur Institute, were used to maintain the infection and as a source of MØ.

Parasites. Amastigotes of *L. amazonensis* LV79 (strain designation MPRO/BR/72/M1841) were obtained from the foot pads of male BALB/c mice as described (14), and suspended in RPMI supplemented with 5% FCS, 50 μg/ml gentamycin, and buffered with 25 mM Mops to pH 7.3. Parasites were kept at 34°C in an air atmosphere and used within 24 h.

Preparation and Infection of MØ Cultures. Elicited MØ were obtained from mice 3–5 d after intraperitoneal injection of 1.5 ml of 1% hydrolyzed starch (Connaught Diagnostics, Toronto, Canada) in PBS. Cell suspensions in PBS (10^5 MØ) were allowed to adhere onto 12-mm diameter coverslips for 10–20 min at room temperature. Coverslips were rinsed with HBSS and incubated in complete medium (37°C, 5% CO_2 in air) in 24-well plates. 24 h later, MØ were infected with 2–3 amastigotes per cell and kept in the same medium for 48 h (34°C, 5% CO_2 in air). For electron microscopy cell suspensions (2 × 10^6 MØ per dish) were allowed to adhere to 35-mm diameter tissue culture plates.

Pulse-chase Particle Transfer Assay. Infected MØ cultures on coverslips were washed twice with HBSS-Mops and 0.4 ml HBSS-Mops-10% FCS added per well. Z, β-glucan, or killed yeast particles suspended in 0.1 ml of HBSS-Mops-10% FCS were added per well for 15–60 min at 34°C at multiplicities of 7–30 particles per MØ. Latex beads and GRC were added, respectively, at 50 μg/ml and 25 GRC/MØ. At the end of the pulse, cultures were twice washed to remove noningested particles and chased in complete medium (34°C, 5% CO_2 in air) for the desired time period. When indicated, FCS was omitted from the medium. At the end of the chase period, cells were washed once with HBSS and fixed for at least 30 min at 4°C in 1.25% glutaraldehyde in PBS. To reduce cell compression during observation, coverslips rinsed in water were inverted in 50% glycerol over 0.15-mm-deep chambers prepared by affixing 9 × 22-mm no. 1 coverslips to standard microscope slides (15). Counts were performed at a magnification of 1,000 with an oil immersion phase contrast objective. At least 200 infected MØ were scored per coverslip, with frequent focusing. The following were estimated: (*a*) the percentage of infected cells in which transfer of at least one particle to at least one PV could be demonstrated; (*b*) the number of particles transferred to PV of infected MØ with transfer; and (*c*) the total number of particles internalized by MØ with transfer. From these values were calculated: (*d*) the number of particles transferred per 100 MØ, and (*e*) the percentage of particles transferred = total no. of particles transferred per MØ × 100/no. particles internalized per MØ with transfer. Coverslips mounted in water were also examined and photographed with a Nomarski differential interference microscope (Olympus, Tokyo, Japan) which provides precise optical sections without the refringent halos associated with phase contrast.

Electron Microscopy. MØ cultures on 35-mm diameter tissue culture dishes were pulsed with Z particles, chased in complete medium, rinsed in HBSS, and processed as follows. Cells were first fixed in 2.5% glutaraldehyde (Sigma Chemical Co.) in 0.1 M cacodylate buffer pH 7.2 containing 0.1 M sucrose, 5 mM Ca^{2+}, and 5 mM Mg^{2+}. After washing overnight at 4°C with the same buffer, cells were postfixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 M cacodylate buffer. Cells were then scraped off the culture dishes with a rubber policeman, concentrated in agar, and treated for 1 h at room temperature with 1% uranyl acetate in veronal buffer at a final pH of 5.0 Samples were dehydrated in a graded series of acetone and embedded in Epon. Thin sections were stained with 2% uranyl acetate and lead citrate.

Results

General Observations. In most experiments *Leishmania*-infected MØ were pulsed for 1 h at 34°C with Z particles, and washed and chased in complete medium for different time periods before fixation. Fig. 1, *A* and *B* show that Z particles could be detected by either phase contrast- or differential interference microscopy within the PV of the infected MØ. This conclusion was supported by transmission electron microscopy (Fig. 2). Fig. 2 *A* displays an infected MØ containing phagocytized Z particles that have not been transferred to the PV. The absence of unoccupied vesicular space suggests that vesicle membranes were tightly apposed to the particles. In addition, regions of close contact were found between Z-containing vesicles and the PV (*arrowheads*). Fig. 2 *B* shows two Z which appear to be free within a PV. Membranes separating the particles from the parasites could not be identified. A vesicular and amorphous material was commonly found within the PV of infected MØ (Fig. 2, *A* and *B*).

Selectivity of Particle Transfer. Heat-killed yeast and β-glucan particles were also transferred to PV. Heat-killed yeasts were larger, more refractile than Z particles, and often presented a distinct cell wall. Once within MØ, their refractility was reduced, presumably by lysosomal degradation (Fig. 1 *C*). β-glucan particles were smaller, less refractile than Z, and tended to form aggregates within PV (Fig. 1, *D* and *E*). In contrast, and confirming earlier preliminary results (10),

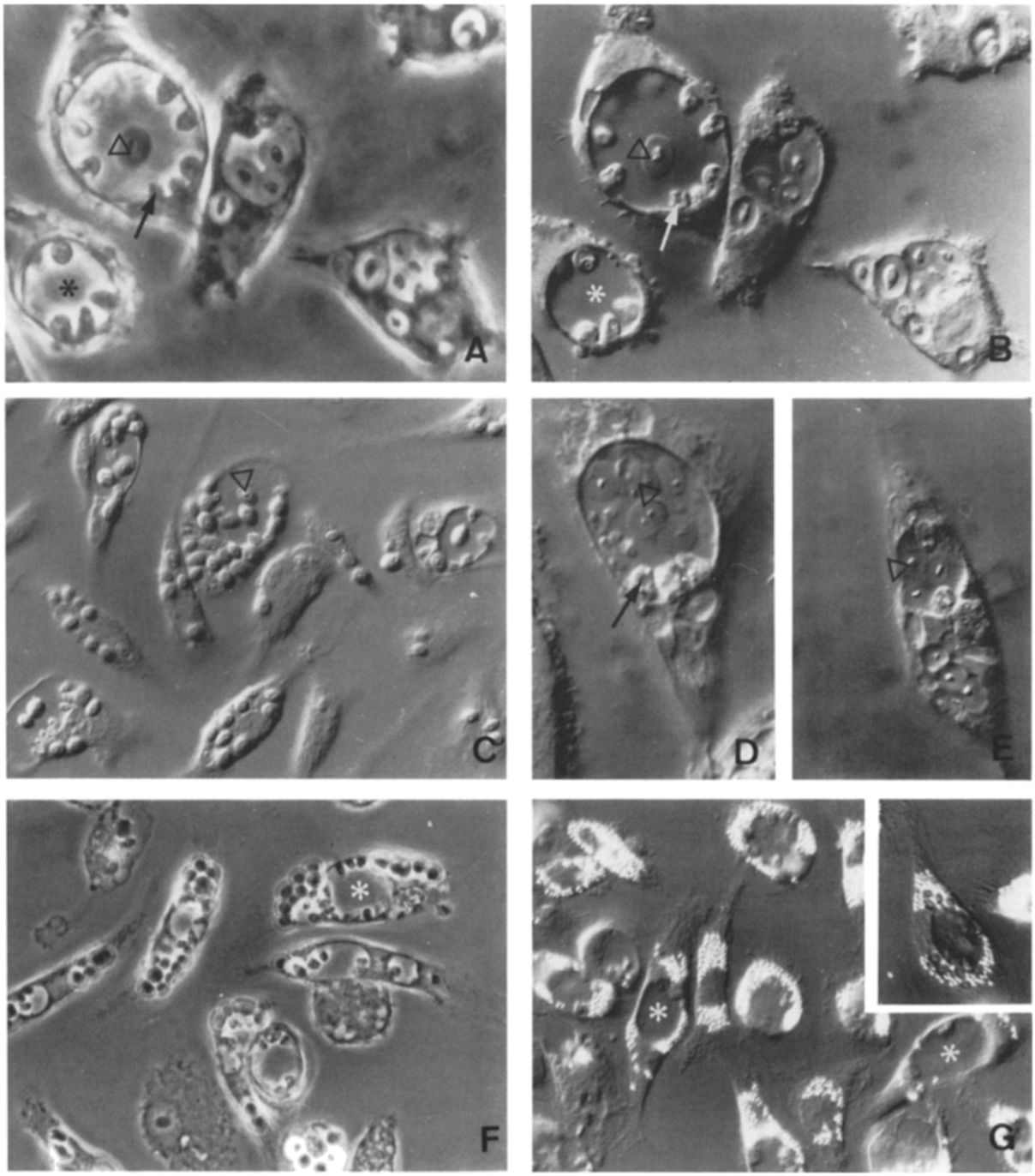
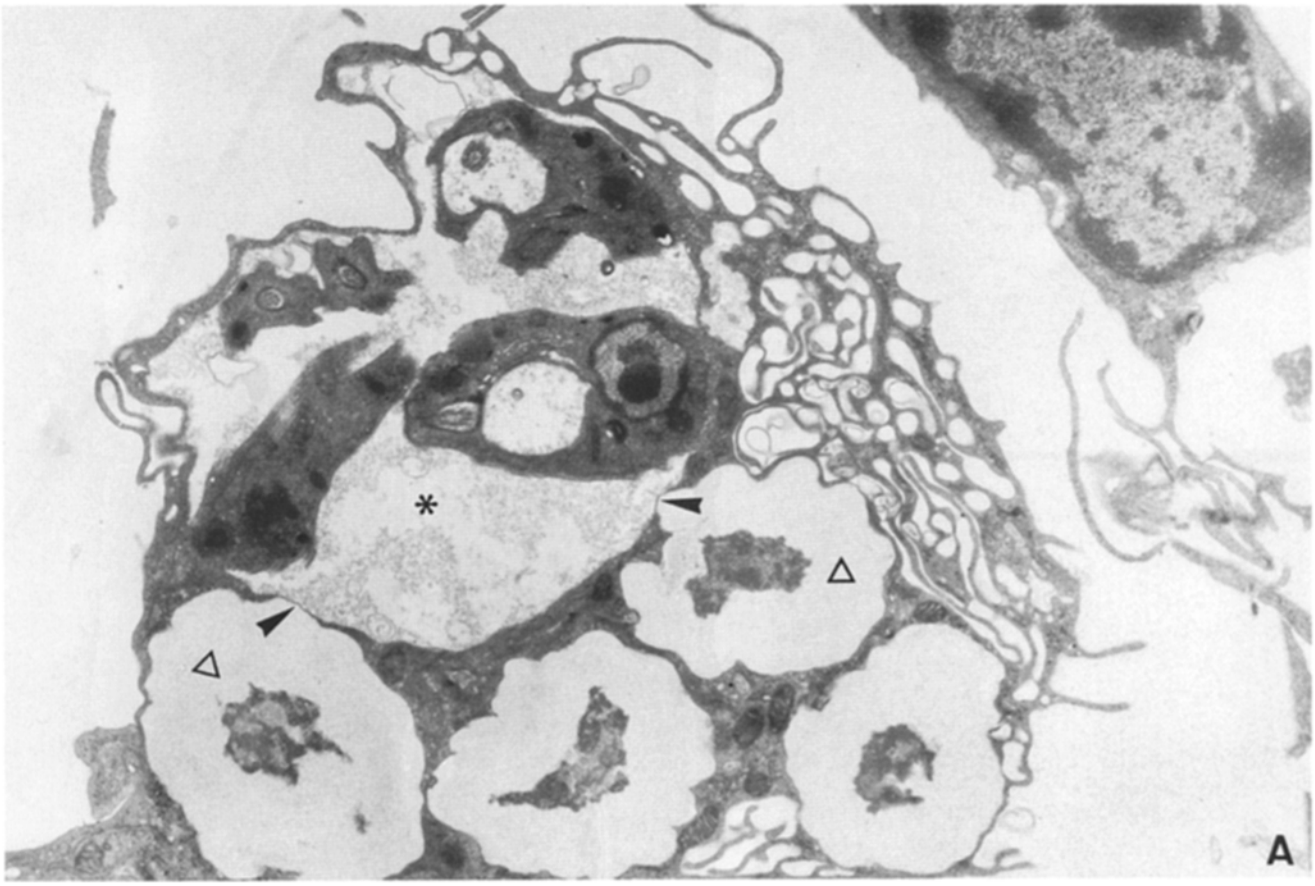


Figure 1. Selectivity of particle transfer to the PV of *L. amazonensis*-infected MØ. (*) PV; (*arrows*) *Leishmania*; (*open triangles*) particles within PV. (*A*) Z particles, phase contrast. (*B*) Same field under interference contrast (×1,500). (*C*) Heat-killed yeast (×1,000). (*D* and *E*) β-glucan particles (×1,500 and ×1,000, respectively). (*F*) GRC (×700). (*G*) 1-μm diameter latex beads (×700); (*inset*) cell from culture incubated with both latex and Z particles. Infected MØ cultures were pulsed for 1 or 2 h with the particles, washed and chased for 24 h (*A* and *B*) or 4 h (*C–G*).

glutaraldehyde-fixed erythrocytes (GRC, Fig. 1 *F*), latex beads (Fig. 1 G), or IgG-coated GRC or fresh red blood cells (not shown) were not transferred to PV. Furthermore, when infected MØ were offered mixtures of latex beads and Z particles, only the latter found their way to PV (Fig. 1 G, *inset*).

Relationship of Transfer to Cell Size and Shape. Transfer of Z was more commonly found in smaller cells, which contained a single large PV surrounded by a rim of cytoplasm, and ingested few particles (Fig. 1, *A* and *B*). Transfer was less frequent in larger, elongated, or polygonal cells which took up more particles and displayed several PV. The reason(s) for these differences need further study, but one explanation is that the path from pseudopod to PV is shorter in the smaller than in the larger cells.

Relationship of Z Transfer to PV Size. Transfer of Z to small vacuoles, defined as those with a diameter less than the length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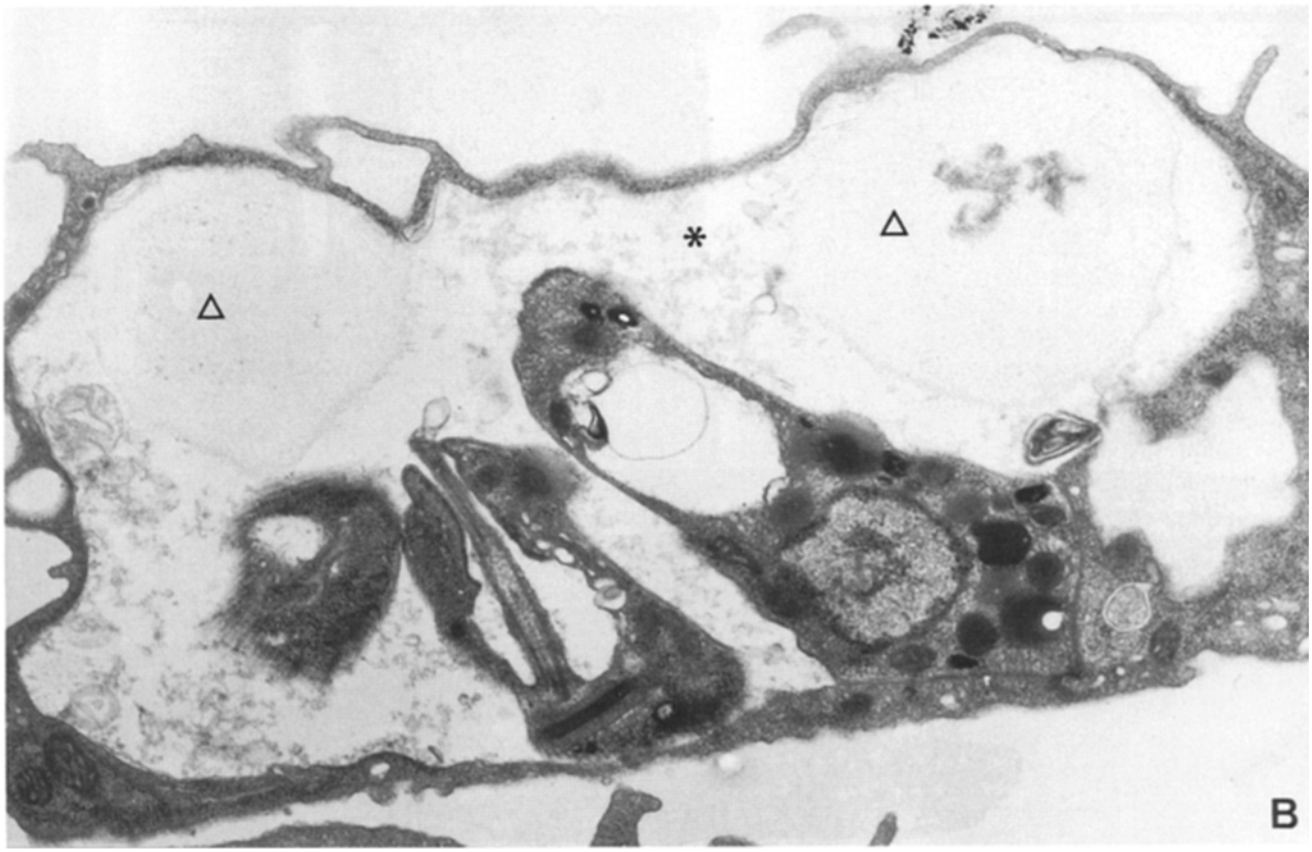
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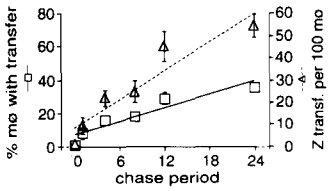

Figure 3. Kinetics of Z transfer. *Leishmania*-infected MØ were pulsed for 1 h with 6 Z/cell and chased from 1 to 24 h in particle-free medium before fixation. The percentage of MØ containing at least one Z in one PV (□) and the total number of Z transferred per 100 MØ (△) were determined as in Materials and Methods. In this and in the following figures, each point gives the average of three coverslips. At least 200 cells were scored per coverslip and SE are indicated by vertical lines.

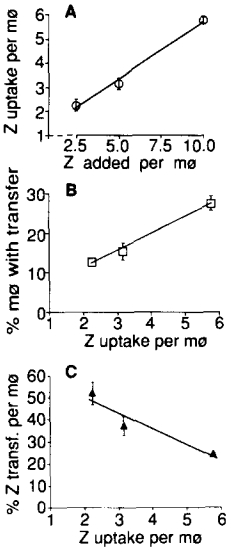

Figure 5. Z transfer to PV as a function of particle uptake (*A*) Relationship between numbers of Z added per cell and the actual number of Z taken up. (*B*) Percentage of MØ with transfer as a function of Z uptake per MØ. (*C*) Z transfer per MØ as a function of Z uptake. 1-h pulse followed by 4-h chase.

of two amastigotes, was rarely found. It was intermediate in medium-sized vacuoles, of diameter equivalent to two to five amastigotes (Fig. 1, *C–E*), and most common in large vacuoles (diameter greater than five amastigotes, Fig. 1, *A* and *B*).

Time Course. For long-term kinetics, infected cultures were pulsed with 6 Z particles per cell, washed and chased for different time periods. Under these conditions, each MØ took up an average of three to four particles, and little transfer to PV was detected at the end of the pulse. Thereafter, transfer increased linearly for up to 24 h, when, in the experiment shown, about 35% of the MØ contained one or more Z in at least one PV (Fig. 3). Higher numbers of Z per cell (30:1) were used to evaluate the early kinetics of transfer. Fig. 4 *A* summarizes such an experiment in which infected MØ cultures were fixed at different times after coincubation with Z particles. Transfer was rare after 15 min and reached 16% of the MØ after 1 h. During this time, the number of Z particles taken up by the MØ with transfer increased from 2.5 at 15 min to about 12 at 60 min (Fig. 4 *B*). In the same experiment, at each time period parallel cultures were rinsed and chased for 2 h in plain medium. Fig. 4 *A* shows that the percentage of MØ with transfer increased during the chase. However, as expected, the numbers of Z per MØ did not change.

Transfer at Different Z Loads. Infected MØ were pulsed for 1 h with an estimated multiplicity of 2.5, 5.0, or 10 Z per cell, and washed and fixed after 4 h chase. These particle inputs resulted in average cell uptakes of 2.1, 3.4, and 6 Z in the MØ with transfer (Fig. 5 *A*). Fig. 5 *B* shows that the percentage of MØ with transfer was linearly related to the numbers of Z taken up per phagocyte. However, as shown in Fig. 5 *C*, the proportion of Z transferred, a measure of the efficiency of the process, decreased as particle loads increased.

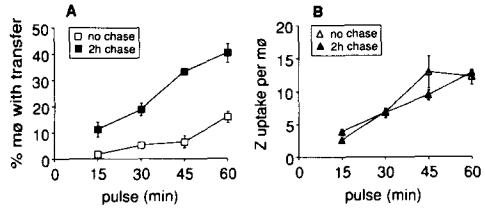

Figure 4. Short term kinetics. Infected MØ pulsed with 30 Z/cell and either fixed immediately (□, △) or chased for 2 h (■, ▲). (*A*) Percentage of MØ with transfer. (*B*) Total Z uptake by the MØ as a function of the duration of the pulse in nonchased and chased cultures.

Temperature Requirement. In the previous experiments, both pulse and chase were performed at 34°C. To examine temperature dependence of transfer, MØ were loaded for 45 min at 20°C, (a temperature at which particle uptake does occur, albeit less effectively), rinsed, and either fixed immediately or chased at 20 or 34°C for 2 or 4 h. In a typical experiment with 20 Z/MØ, no transfer was detected at the end of the pulse. After periods of chase of 2 and 4 h at 20°C, the percentages of MØ with transfer were, respectively, 4.8 ± 0.7 and 6.0 ± 1.1 (mean ± SE of triplicate coverslips). At 34°C, the corresponding figures were, respectively, 8.8 ± 1.7 and 19.2 ± 1.8. Thus, although some transfer was obtained at 20°C, transfer was clearly higher at 34°C.

Effect of Agents Known to Induce Vacuolar Alkalinization. The percentage of MØ with transfer was increased by monensin, ammonium chloride, and chloroquine (CQ) (Figs. 6–8). These agents, particularly CQ, increased the size of PV and induced MØ vacuolization. The latter was more noticeable in noninfected than in infected cells. Monensin and ammonium chloride did not increase the numbers of Z transferred per MØ, and did not appear to damage intracellular amastigotes. In contrast, CQ increased the number of Z transferred per MØ and killed many parasites when used at a concentration of 50 μM for 2 or 4 h. To examine if the effect of CQ continues after the drug is removed from the medium, the following

Figure 2. Electron micrographs of *L. amazonensis*-infected MØ after uptake of Z particles. (*A*) 1-h pulse with Z (15 particles/cell) followed by 24-h chase. (*) PV containing *Leishmania* amastigotes. (△) Z particle. No transfer of the particles to the PV is shown, but two particles show close apposition to the membrane of the vacuole (◀) (×13,000). (*B*) 1-h pulse with Z followed by 1-h chase. Two Z particles are shown within the PV (×21,000).

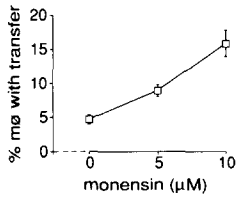

Figure 6. Effect of monensin on the transfer of Z to the PV. MØ cultures pulsed with 6 Z/MØ, washed and chased for 60 min in the presence of the concentrations of monensin indicated. Values corrected for transfer of Z in cultures fixed at the end of the pulse.

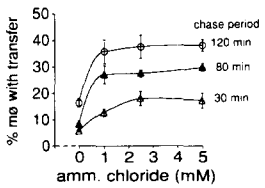

Figure 7. Effect of ammonium chloride on Z transfer to PV. MØ cultures pulsed with 7 Z/cell and chased with different concentrations of NH_4Cl for the time periods indicated. Values corrected as Fig. 6.

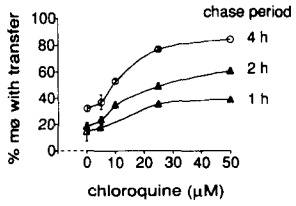

Figure 8. Effect of CQ on the transfer of Z to PV. MØ cultures pulsed with 7 Z/cell for 45 min, chased for 15 min to allow for internalization of bound particles, and incubated for the times indicated with different concentrations of CQ. Values corrected as in Fig. 6.

experiment was performed. MØ cultures were pulsed with Z particles and chased for 2 or 4 h in medium alone or with the addition of 20 μM CQ. Other cultures were incubated with CQ for 2 h, and washed and chased for another 2 h in the absence of the drug. The results, summarized in Fig. 9, show that removal of the drug reduced the rate of transfer of Z into the PV.

Requirement for Actin Polymerization. Transfer was reproducibly inhibited by cytochalasin D given during chase (Fig. 10), suggesting that actin polymerization is directly or indirectly required for the translational movement of Z before they reach the PV. In contrast, transfer of Z was unaffected by treatment with the antitubulin nocodazole which was given at 1 μM for 18 h and was continued throughout the pulse and chase periods.

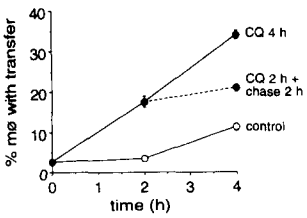

Figure 9. Effect of removal of CQ from the medium on the transfer of Z to PV. MØ cultures pulsed for 45 min with 15 Z/cell were fixed after a 2- or 4-h chase in the presence of 20 μM CQ (——, ●) or in plain medium (○). Other cultures were chased for 2 h in the presence of CQ, washed and chased again for 2 h in plain medium (- - - -). SE were close in size to the symbols.

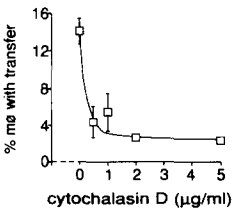

Figure 10. Effect of cytochalasin D on the transfer of Z particles to PV. MØ pulsed with 15 Z/cell, and chased for 4 h in complete medium alone, or containing the indicated concentrations of cytochalasin D.

Discussion

We have confirmed an earlier incidental observation that Z particles can be transferred to the PV of *Leishmania*-infected MØ (9). We have also established a light microscopic procedure to quantify the transfer, and delineated several features and requirements of the process.

Transfer Assays. In most experiments, infected MØ cultures were pulsed with Z particles for 60 min, washed, chased for different time periods in particle-free medium, and fixed for microscope counts. This design permits the separation of phagocytosis, which takes place during the pulse, from particle transfer to PV, which mainly occurs during the chase period. However, since transfer requires that donor vesicles and recipient PV be close to each other, translational movement of the former should be a necessary component of the overall process. This consideration is relevant to the interpretation of several of the experiments reported here. For instance, the kinetics of transfer (Figs. 3 and 4) may not mainly reflect the time needed for actual particle transfer, but rather that required for translocation of donor vesicles. Similarly, the inhibition by cytochalasin D of the transfer of Z to PV (Fig. 10) may be explained by the involvement of an actin motor in the translocation of Z vesicles, rather than in the Z transfer event (16, 17).

Particle Selectivity. The pioneering studies of Oates and Touster (18, 19) on *Acanthamoeba* demonstrated and quantified in intact cells and in cell homogenates the nonselective fusion of particle-containing phagocytic vesicles. In the present experiments, transfer of the same particulate markers to MØ PV was apparently selective, since yeast-derived particles were transferred, whereas latex beads or aldehyde-fixed erythrocytes were not. The mechanisms that account for this selectivity are not understood. Yeast-related particles display a high density of carbohydrate ligands, and thus can be expected to induce receptor aggregation, known to be important in the transfer of molecular or particulate ligands to lysosomes (20). Furthermore, the relative resistance of yeast polysaccharides to lysosomal degradation may extend the life of the ligand–receptor complexes. A second possibility is that transfer is dictated by the nature of the receptors that recognize the particles. Different receptors may account for the direct recognition of yeast particles by MØ. They are the mannose-fucose receptors (21, 22), the lectin-binding site of the α chain of CR3 (CD11b-CD18) (23), and the β-glucan receptor (24, 25). It is possible that the cytosolic tails of one or more of these receptors contain signals that trigger the interaction of donor vesicles with PV (26). Lastly, selectivity of transfer may depend on different rates of progression of the particles along the endocytic pathway, i.e., whether particles are contained in phagosomes, in phagolysosomes, or in more complex organelles (27). Although Z, as well as reduced and alkylated, nonopsonized or opsonized yeast particles are competent inducers of phagosome lysosome fusion (28, 29), MØ infected with *L. amazonensis* are depleted of cytochemically

or ultrastructurally identified lysosomes (30). It is thus possible that the kinetics of phagosome-lysosome fusion may be slower in infected than in control MØ. This could make more phagosomes available for interaction with PV.

Mechanisms of Transfer. We have as yet no information on the mechanisms of delivery of yeast-derived particles to PV. Such delivery may involve fusion of PV with the donor vesicles, but a mechanism analogous to phagocytosis, involving attachment and invagination of donor vesicles into PV cannot be excluded. Indeed, since internalized membranes could be digested during the chase periods, both mechanisms could result in similar ultrastructural features (18). The second mechanism is compatible with earlier cinematographic observations that the large PV are easily deformable (11), and with the present finding that Z particles were more frequently transferred to large than to small PV. Furthermore, light microscopic observations of "herniation" of Z particles into PV, and of, albeit infrequent, "mesentery-like" phase dense connections between the Z particles and the inner face of the PV, may be best explained by a vesicle invagination mechanism (P. Veras and M. Rabinovitch, work in progress).

Enhancement of Z Transfer by Compounds Known to Increase the pH of Acidified Organelles. The ionophore monensin, and the weak bases NH_4Cl and CQ enhanced in time- and concentration-dependent fashion the transfer of Z to PV (Figs. 6–9). Ionophores and acidotropic compounds have been shown to reduce both the intracellular dissociation of receptor–ligand complexes, and the recycling of certain receptors to the plasma membrane, and to inhibit the delivery of the complexes to lysosomes (31). It is thus possible that alkalinization increased the number of receptors associated with the donor vesicles, and therefore the postulated signals associated with the cytosolic tails. The possibility that vacuolar swelling alone promoted or enhanced the interaction of PV with donor vesicles is not compatible with the observations that: (*a*) CQ did not induce the transfer of latex beads or of aldehyde-treated erythrocytes to PV (not shown); (*b*) addition of 50 mM sucrose 24 h before and during the chase period induced MØ vacuolization, but did not enhance transfer of Z to PV (not shown); and (*c*) studies in progress have shown that D-Leu-OMe, an amino acid ester which is only slowly hydrolyzed in lysosomes and does not induce detectable vacuolization of macrophages, markedly enhanced the transfer of Z particles to PV.

It was recently reported that ammonium chloride, which suppresses phagosome-lysosome fusion, increased the fusion of endosomes containing Lucifer yellow or cationized ferritin, to yeast-containing phagosomes of MØ (32). Culture conditions were different from those reported here and live yeasts were used as test particles. These findings raise the possibility that in our experiments NH_4Cl induced the accumulation of Z-containing phagosomes particularly prone to fuse with PV. However, in Hart and Young's experiments, CQ did not increase the fusion of yeast phagosomes with endosomes.

Recent studies documented phagosome-endosome fusion in vivo and in isolated vesicle preparations (33) and endosome–lysosome interactions have been reconstituted in a cell-free system (34). Several requirements for phagosome-endosome fusion such as for ATP, GTP, cytosolic factors, and GTP binding proteins, have now been defined (35, 36). The transfer of particles to the *Leishmania* PV in MØ offers a model which may permit the study of the mechanisms involved in the movement and interactions of large endocytic vesicles, and of their modulation by host-cell or parasite-derived signals, as well as by exogenous effector molecules and pharmacological agents.

Eduardo Cesar Santos Lima, then a medical student at the University of Bahia (Salvador, BA, Brazil), participated in the first light microscopic studies in December 1990.

P. Veras is the recipient of a fellowship from Conselho Nacional de Pesquisas, sponsored by the Graduate Program of the Instituto Oswaldo Cruz (Rio de Janeiro, Brazil). The authors thank Mr. J. C. Benichou and Mr. J. H. Panaud of the Pasteur Institute for printing the photographs. This work was supported by the Centre National de la Recherche Scientifique, Institut Pasteur, and by the Commission of the European Communities Program "Science and Technology for Development" (Contract TR2-0110-F). C. D. Chastellier is supported by INSERM (CJF 90-04).

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Received for publication 13 April 1992.

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