

LEISHMANIA DONOVANI

Hamster Macrophage Interactions in Vitro: Cell Entry, Intracellular Survival, and Multiplication of Amastigotes*

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Visceral leishmaniasis or kala azar is a debilitating, often fatal disease of man, resulting from the infection by a trypanosomatid flagellate protozoan, *Leishmania donovani*. The amastigote stage of this organism parasitizes exclusively the monocyte-macrophage-histiocyte series of the reticuloendothelial system in mammalian hosts. How these parasites gain entrance, survive, and multiply in these mononuclear phagocytes, which are potentially hostile to any invading micro-organism, are questions of importance in the pathogenicity and host immunity of this disease (1). A feasible approach to study these fundamental questions is the use of an in vitro system, by which host-parasite cellular interactions can be closely observed under more defined conditions. Since hamsters serve as an excellent animal model for human visceral leishmaniasis, their macrophages are obviously the host cells of choice for such studies. *Leishmania donovani*-hamster macrophage interactions in vitro have been the subject of investigations by several authors with promastigotes (2-6) or amastigotes (7-9). All these studies, however, either were limited to observations of short duration or yielded inconsistent results, apparently due to the difficulties of keeping host cells adequately in vitro. We now employed an improved method for the satisfactory maintenance of hamster macrophages to explore the full potential of this in vitro system for studying visceral leishmaniasis. In several preliminary reports, we have characterized the adhesion of promastigotes (10) and the mode of their entry into these cells as well as the possible intracellular survival mechanism of amastigotes (11). In this paper, we report amastigote-macrophage interactions with respect to the mechanism of entry, intracellular survival and multiplication in full details with additional evidence to extend our earlier findings reported in brief form (12).

Materials and Methods

Parasites. Strain 1-S of *Leishmania donovani* clone 2 D was used for the present study. The organism was routinely passaged in Syrian Golden hamsters (males, 70-80 g, Lakeview, N. J.) by intracardiac inoculation every 1-2 mo. Amastigotes were isolated in Hanks' balanced salt

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solution (HBSS)¹ or macrophage culture medium (12)² according to the procedure previously described (13). In brief, heavily infected spleens were ground in TenBroek type glass homogenizers; after repeated washing in HBSS by centrifugation at low and high speed, a final amastigote reaction free of intact host cells was obtained. The number of amastigotes was estimated in a bacterial counting chamber or by differential counting referring to a known number of erythrocytes in Giemsa stained preparations.

Hamster Macrophages. Cells used were isolated and cultured from peritoneal cavities with or without prestimulation with 4% thioglycollate broth or culture medium according to the method briefly described before (12). HBSS with 25 mM Hepes or culture medium was injected into peritoneal cavities stimulated 2 days earlier. Peritoneal fluid was then withdrawn, pooled, centrifuged, and resuspended in culture medium. Cell suspension in 0.1–0.3 ml portions each containing 10⁶ cells was distributed to flying coverslips (18 or 22 mm²) in Petri dishes. Cultures were incubated at 37 ± 1°C in 5% CO₂ in air for 2–4 h for cell adhesion and then flooded with additional medium. Before use, coverslips with cell monolayers were rinsed with warm buffer or medium to remove nonadherent cells. Further details on the cultivation of hamster peritoneal macrophages will be described elsewhere.²

Infection Experiments. Amastigotes used were freshly isolated materials kept at 4°C or those incubated for 3–5 h at 27 or 37°C, after which the host membranes around the amastigotes were lost (14, 15). For infection, the hamster macrophages cultured in vitro on coverglasses for 1–7 days were transferred to fresh Petri dishes and covered with 0.1–0.3 ml of hamster macrophage culture medium containing appropriate numbers of amastigotes to produce parasite/macrophage ratios of 2, 10, 20, or 40 to 1. After incubation at 37°C with 5% CO₂ in air for about 4 h, coverslips were rinsed with warm medium to remove free parasites and then transferred again to fresh dishes containing 2–3 ml of the culture medium. The medium was changed every 2–3 days. Samples in duplicates or triplicates were taken at various times up to 4 wk after infection for microscope examinations.

Enumeration of Intracellular Parasites. The coverslips with infected macrophages were dried, fixed in absolute methanol, and stained with Giemsa. Infectivity or the percent of infected macrophages containing at least one parasite and multiplication index or the average numbers of amastigotes per cell were determined by examining at least 200 and 100 macrophages per coverslip, respectively. The total numbers of determinations and the standard deviations for each variable are shown in Table I.

Electron Microscopy-Enumeration of Lysosome-Phagosome Fusion. The method for electron microscopy was already described (12). The secondary lysosomes of hamster macrophages were labeled with thorotrast by a modified method (12) of Jones and Hirsch (16). The labeled macrophages, after an appropriate period of incubation, were infected with amastigotes at a parasite/macrophage ratio of 2 or 10 to 1. Samples were removed at different times after infection and processed for electron microscope observations. The percent of parasitophorous vacuoles containing thorotrast granules was calculated in each sample by examining at least 200 different vacuoles. The dense marker was also fed to the macrophage cultures infected with amastigotes for different periods of time to determine if it might be incorporated into the parasitophorous vacuoles.

Results

Infectivity and Multiplication of Amastigotes in Hamster Macrophages—Light Microscopy. No apparent differences in the rate of ingestion and growth of amastigotes were observed with parasites with or without host membranes in macrophages, which were aged in vitro for various times (1–7 days), stimulated or unstimulated in vivo with thioglycollate broth or culture medium. The experiments with unelicited macrophages were terminated in 5–7 days when an accurate quantitation of amastigotes became difficult due to the

¹ Abbreviation used in this paper: HBSS, Hanks' balanced salt solution.

² K-P. Chang. 1978. Hamster peritoneal macrophages in vitro: substratum adhesion, spreading, phagocytosis, and phagolysosome formation. Manuscript submitted for publication.

TABLE I
Infectivity and Multiplication of Leishmania Donovanii Amastigotes in Hamster Peritoneal Macrophages

Parasite/macrophage ratio	Exp. No	Infectivity*		No. parasites† per cell		Multiplication‡	
		1 day	7 day	1 day	7 day		
		%					
2:1	7	45.9 ± 9.0	46.6 ± 5.6	1.7 ± 0.4	6.6 ± 1.2	4.0 ± 0.7	
20:1	4	94.0 ± 2.5	94.0 ± 6.0	11.3 ± 1.8	37.3 ± 7.4	3.4 ± 1.4	
40:1	3	99.0 ± 1	99.0 ± 1	32.7 ± 3.3	70.0 ± 11.9	2.1 ± 0.2	

* Percent of infected macrophages determined by counting at least 200 cells in each sample.

† Average numbers of parasites per macrophage determined by counting at least 100 cells in each sample.

‡ Fold increase in parasite number per macrophage from day 1 to 7.

|| Mean percent or parasite number per macrophage ± SD.

extensive loss of cells. The following observations are applicable to all experiments, irrespective of the "forms" of amastigotes or the "state" of host cells specified above. During the first 4 h of infection period, there was a gradual increase in percent of macrophages infected and in parasite numbers per cell in all experiments with either low or high parasite/macrophage ratios, indicating the uptake of amastigotes by the host cells. The kinetics of infection varied with the parasite/macrophage ratios: at a high ratio of 2 to 1, the uptake began to reach a plateau after 4 h when about 40–45% of macrophages contained about two parasites in each. From day 1 to 7 after infection, an increase in the average numbers of amastigotes per cell was consistently observed in a total of 14 experiments with three different parasite/macrophage ratios (Table I). Both infectivity and multiplication of parasites began to decline in 2–3 wk, depending on the parasite/macrophage ratios used, and they eventually disappeared in the macrophage cultures after incubation for 4 wk *in vitro*. Under no circumstance was disruption of the macrophages due to parasite multiplication observed.

Light microscope studies showed that amastigotes occurred individually or in clusters (not shown) in the cytoplasm of infected cells (Figs. 1–3), essentially identical in cytological features to those found in infected spleens. The numbers of amastigotes varied widely with individual cells, which were either filled with numerous parasites at a high parasite/macrophage ratio (Fig. 3) or only lightly infected with no more than several parasites per cell at a low ratio (Fig. 1). During the postinfection period, some of the parasites were seen to lie against the inner demarcation of huge parasitophorous vacuoles (Fig. 3). Dividing forms of amastigotes became abundant in day 3–7 after infection (Fig. 3), in agreement with the quantitative data obtained. After 1 wk *in vitro*, amastigotes appeared to be much more numerous in some macrophages than in others with about the same number of parasites as in day 1, suggesting that the parasites in the latter persisted but did not grow (compare Figs. 1 and 2). The general impression was that there were variations among individual macrophages in supporting the growth of the parasites.

Host-Parasite Cellular Interactions—Electron Microscopy. The ultrastruc-

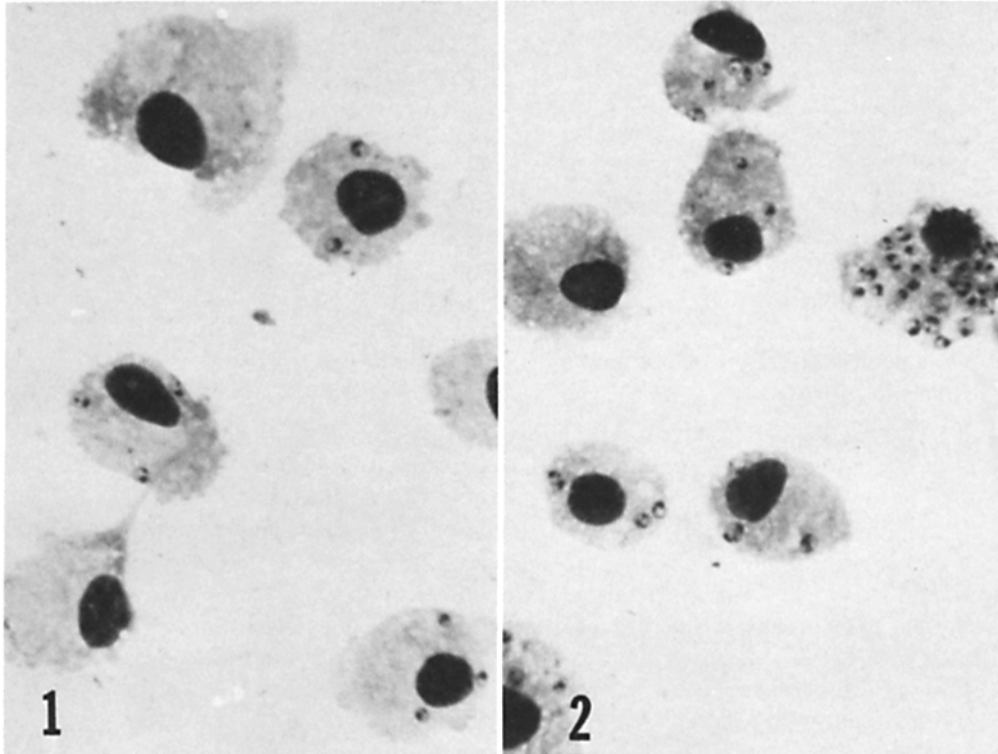


FIG. 1. Representative portion of a macrophage culture infected with amastigotes for 1 day at low parasite/macrophage ratio of 2:1. Note the parasite number per cell which is rarely greater than five. Giemsa stain. $\times 800$.

FIG. 2. A representative portion of a macrophage culture infected with amastigotes at low parasite/macrophage ratio of 2:1 for 7 days. Note the presence of macrophages with only 2 or 3 parasites as well as the one with more than 20 parasites. Giemsa stain. $\times 800$.

ture of amastigotes cultured in hamster macrophages showed no apparent changes during a period of 2 wk *in vitro* and was essentially identical to that described by others from samples obtained *in vitro* (5) or *in vivo* (17, 18). Nor was there any change in ultrastructure of macrophages or orientation of their cell organelles associated with the infection (except for the presence of parasitophorous vacuoles). During the early infection period, amastigotes were seen to be sequestered by macrophage pseudopodia or cytoplasmic extensions devoid of cell organelles (Fig. 4) and then became interiorized into loose phagosomes (Fig. 5). This process of parasite ingestion was identical in experiments with amastigotes enclosed by the original host membranes or those treated to strip these layers. When present, these membranes remained intact during the engulfment phase (Fig. 4), began to disintegrate soon after the internalization of amastigotes (Fig. 5), and eventually disappeared upon further incubation (Figs. 6-10). There was no evidence of disruption of phagosome membranes due to the parasite activity at any stage of the infection. After incubation for longer than 1 day *in vitro*, the loose type phagosomes containing amastigotes seen earlier gave way to three types of parasitophorous vacuoles which were

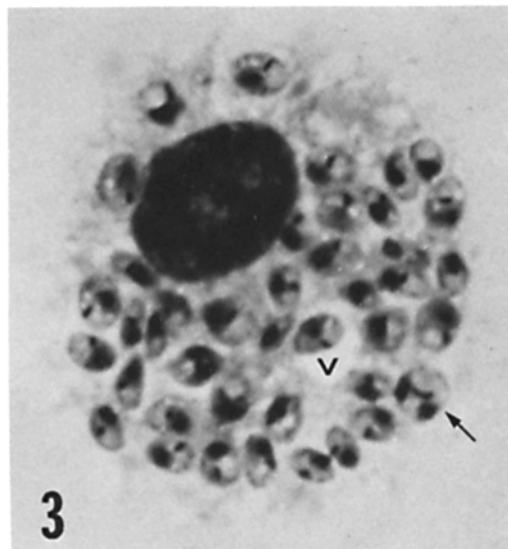
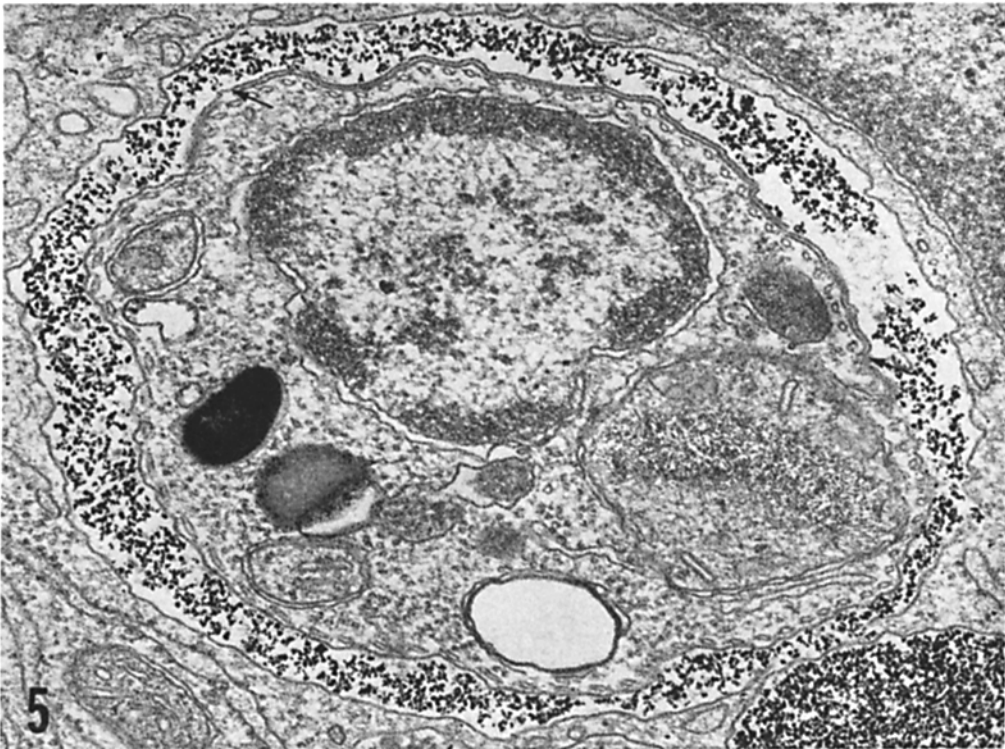


FIG. 3. Macrophages infected with amastigotes at high parasite/macrophage ratio of 10:1 for 3 days. Note the numerical abundance of parasites per cell, the occurrence of dividing forms (arrow), and the partial adhesion of amastigotes to the inner demarcation of a distended vacuole (v). Giemsa stain. $\times 2,000$.

categorized according to the spacial arrangement of parasitophorous vacuoles which were categorized according to the spacial arrangement of parasites in relation to the host vacuolar membranes. The first type had amastigotes surrounded by tight fitting host membranes, leaving virtually no vacuolar space (Fig. 6). The second type was the case where amastigotes adhered in part to the vacuolar membranes, with the remaining portions being free in distended vacuoles of varied sizes and filled with electron dense amorphous materials (Figs. 8 and 9). Less frequently observed was the third type, in which case amastigotes were located in large vacuoles, being well separated by a vacuolar space from the host membranes (Fig. 10). All three types of parasitophorous vacuoles have been encountered in the same cell. Also present were forms intermediate between type 1 and 2 (Fig. 7). Type 2 and 3 vacuoles frequently contained more than one amastigote as well as dividing forms, as indicated by the duplication of cell organelles, such as flagella, nuclei, and/or kinetoplasts (Figs. 10 and 11).

Phagosome-Lysosome Fusion during the Infection. The sequential events that led to the ultimate fusion of parasitophorous vacuoles with secondary lysosomes were reconstructed from examination of samples by transmission electron microscopy at different times after entry of amastigotes into the thorotrast-labeled macrophages. During the engulfment stage, amastigotes were trapped by the pseudopodia of macrophages in early phagosomes, which were devoid of the dense label, indicating that phagosome-lysosome fusion had not yet occurred (Fig. 4). Only when amastigotes became interiorized into the cytoplasm of the macrophages, did the thorotrast granules appear in the loose parasitophorous vacuoles, indicative of their fusion with secondary lysosomes



(Fig. 5). From 1 to 4 h after infection, the vacuoles containing both amastigotes and thorotrast granules increased from 50 to 80% of the total vacuoles examined (Table II) and were of the loose type (Fig. 5). During the postinfection periods from day 1 to 7, thorotrast marker was present in about 60% of the parasitophorous vacuoles (Table II), which were of type 2 and 3 described above (Figs. 8-10). Thorotrast granules were absent in the tight-fitting parasitophorous vacuoles of type 1 with host and parasite membranes lying in close apposition (Fig. 6). When added to macrophage cultures already infected with amastigotes *in vitro* for several days, thorotrast granules appeared on the surface of the macrophages, in pinosomes, secondary lysosomes and all, except type 1, parasitophorous vacuoles (Fig. 11).

Thorotrast granules were also observed in flagellar pockets and dense cytoplasmic granules of amastigotes after infection for 7 days (Fig. 9) suggesting that the dense label was ingested by the parasites.

Discussion

The present study provides quantitative evidence indicating an avid ingestion of *L. donovani* amastigotes by hamster macrophages. The fact that the uptake occurs in the absence of specific opsonization implies that it may not involve receptors for immune ligands on the surface of the macrophages. A similar suggestion has been advanced for the entry into mouse macrophages of *Trypanosoma cruzi* supported by stronger evidence (19). The present study revealed no difference between "naked" and "membrane-bound" amastigotes in their entry into hamster cells, indicating that the host membranes around the isolated parasites must be recognizable just as well by macrophages as the parasite surface. Whether this may indicate certain alterations of the host membranes by the intracellular parasites is not clear, but such modifications of different nature have been proposed (1) and supported by experimental evidence (16). The significance of this finding also remains elusive until further study is made to determine which of the two amastigote forms normally occurs during its uptake by macrophages *in vivo*. The ingestion of amastigotes by hamster macrophages follows the same sequential events described for phagocytosis of other intracellular parasites (20, 21). Endocytic activity of the macrophages appears to be the primary agency for amastigotes to gain intracellular entrance—a view supported by their inability to invade nonphagocytic

FIG. 4. Portion of a thorotrast-labeled macrophage infected for 30 min showing the trapping of a "membrane bound" amastigote by cell pseudopodia in an early phagosome. Note the presence of a thorotrast-filled vacuole, presumably a secondary lysosome (SI) adjacent to the phagosome containing the parasite. The absence of the dense label in this vacuole indicates that phagosome-lysosome fusion has not yet occurred. The host-membrane around the parasite remains intact at this stage. The absence of thorotrast granules on the cell surface and the pinosomes indicates the specificity of the labeling. $\times 45,000$.

FIG. 5. Portion of a thorotrast-labeled macrophage infected for 30 min showing an interiorized amastigote in a phagosome with thorotrast granules indicating its fusion with secondary lysosomes. Note the partial dissolution of the "host membrane" adhered to the parasite. Arrows indicate the points from where the adherent "host membrane" disintegrated. $\times 56,000$.

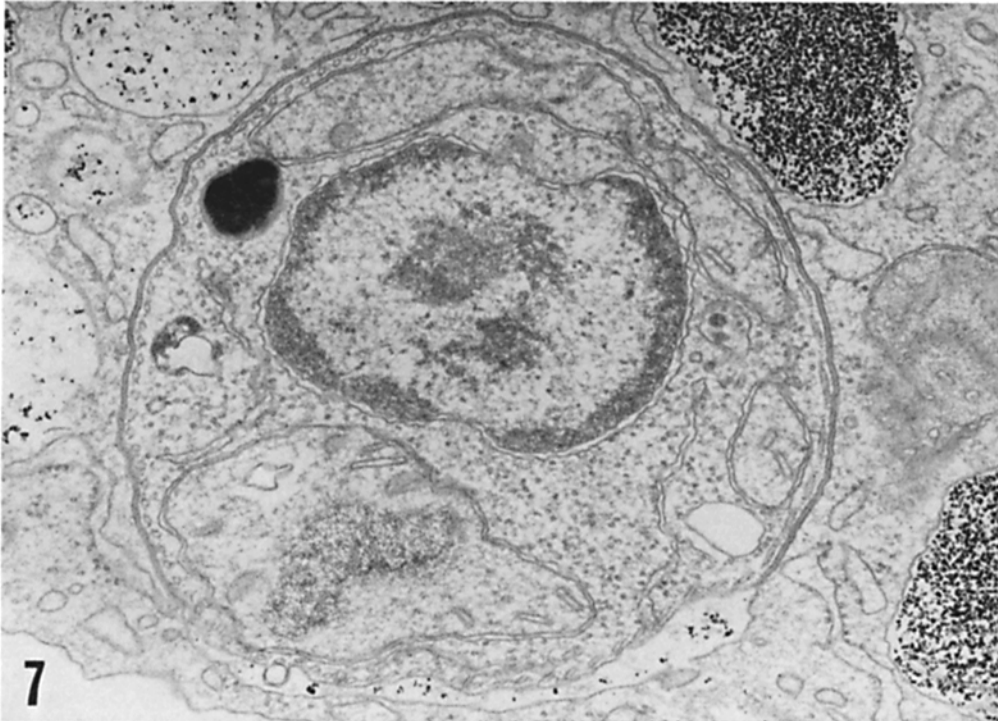


FIG. 6. Portion of a macrophage infected for 3 days showing a type 1 parasitophorous vacuole. The host and parasite membranes are in close apposition, leaving no vacuolar space in between or room for thorotrast granules. $\times 47,000$.

FIG. 7. Portion of a thorotrast-labeled macrophage infected for 3 days showing the form of parasitophorous vacuole intermediate between type 1 and 2. The host and parasite membranes are mostly closely apposed, but separated in one portion where a few thorotrast granules are visible in the vacuolar space formed. $\times 47,000$.

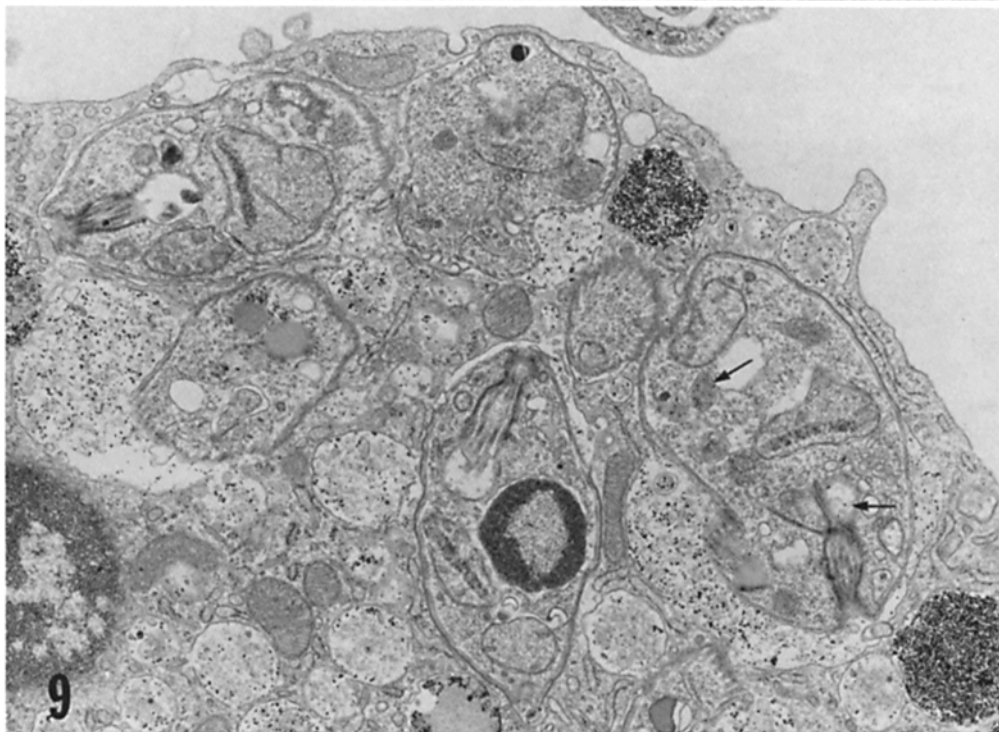


FIG. 8. Portion of a macrophage infected for 3 days showing a type 2 parasitophorous vacuole. The amastigote appear to "push into" host cytoplasm at one portion of the vacuole, leaving the opposite end free in the large vacuolar space filled with amorphous electron dense materials. $\times 27,000$.

FIG. 9. Portion of a macrophage infected for 7 days in vitro showing four amastigotes mostly in type two parasitophorous vacuoles. Arrows indicate the labeled cytoplasmic dense granules and flagella pockets of the parasites, indicating that they ingest thorotrast granules. $\times 17,000$.

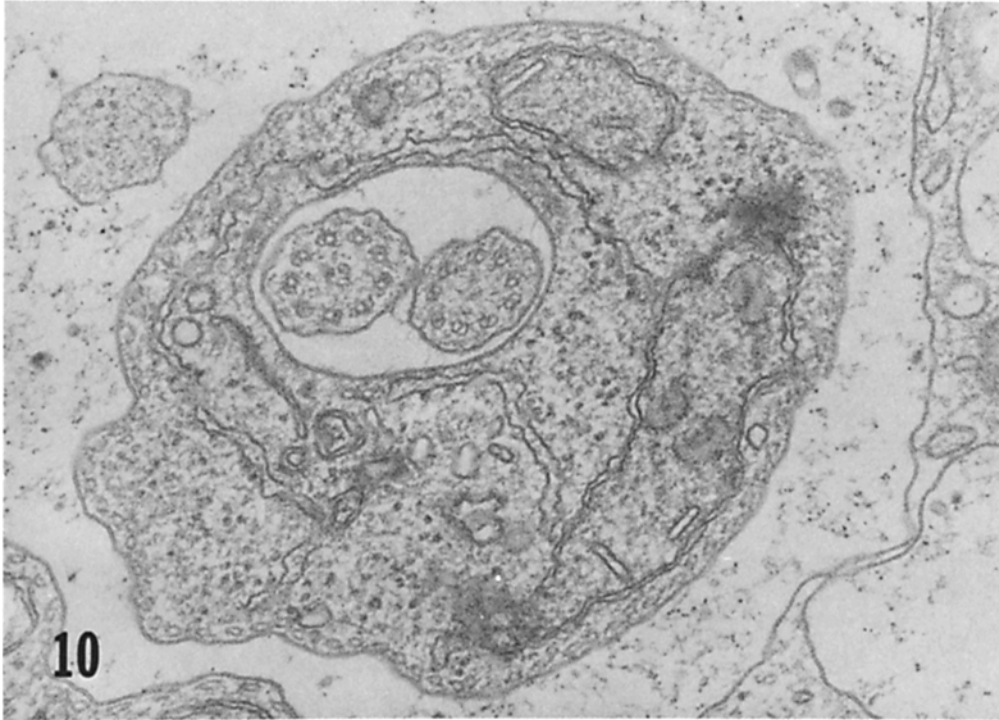


FIG. 10. An amastigote lying in a large type 3 vacuole of a macrophage infected for 3 days. Note the absence of any contact of the parasite with the vacuolar membrane and the presence of two flagella in the flagellar pocket, indicative of cell division. $\times 6,600$.

cells, such as hamster fibroblasts (7). Indeed it would be rather redundant for *L. donovani*, as an obligate intracellular parasite of phagocytes, to evolve a mechanism for cell entry instead of exploiting the normal activity of the host cells. This general concept, however, does not exclude the possibility that leishmania may gain access into nonphagocytic cells, as do the promastigotes of *Leishmania braziliensis* into human skin fibroblasts apparently by "stimulated phagocytosis" (22).

Based on the thorotrast labeling method, quantitative evidence is presented to support our earlier findings that lysosome-phagosome fusion occurs after the entry of *L. donovani* amastigotes into hamster macrophages (12). Employing a different labeling technique for the study of *Leishmania mexicana*-mouse macrophage interactions, Alexander and Vickerman (23) reached a similar conclusion, though no quantitative data was presented. Moreover, the present study establishes the sequence of the fusion events which does not occur during the early engulfment stage but after the completion of the internalization phase, in agreement with the normal process of intracellular digestion of foreign objects. In phagolysosomes, amastigotes maintain their ultrastructural integrity for up to 2 wk in contrast to the adherent host membranes, which disintegrate much sooner than otherwise expected. Evidently, hydrolytic enzymes in these parasitophorous vacuoles must be functional to degrade the nonparasite membrane material, lending support to our earlier suggestion (12)

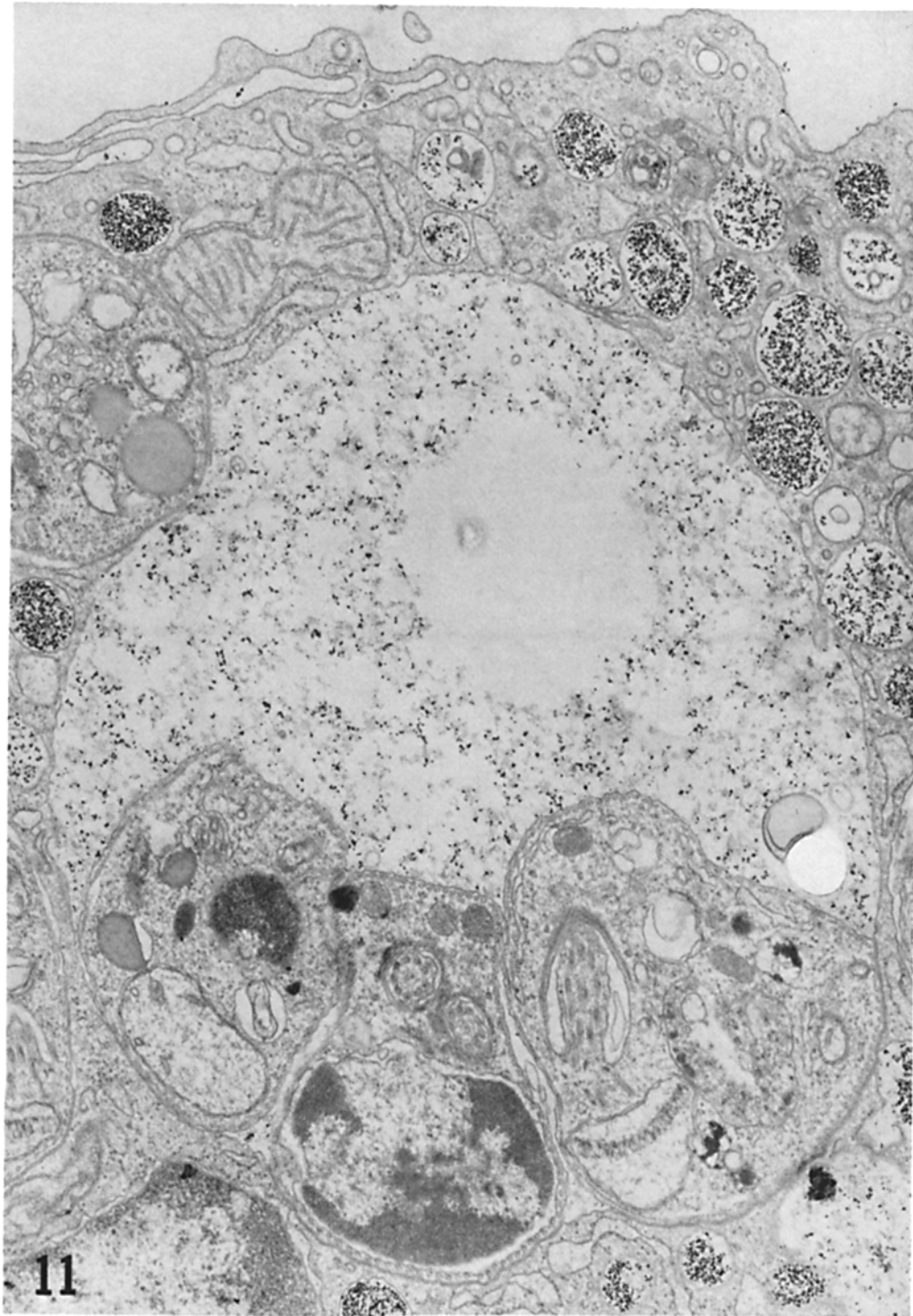


FIG. 11. Portion of a macrophage infected for 3 days and labeled with thorotrast for 16 h showing the entry of the dense label into a large parasitophorous vacuole apparently through the cell surface, pinosomes, and secondary lysosomes. Three amastigotes lie closely together against the inner lining of the parasitophorous vacuole. The amastigote in the middle with 2 flagella and condensed chromatin materials in the nucleus is apparently undergoing cell division. $\times 28,000$.

TABLE II
*Secondary Lysosomes Containing Amastigotes after Infection of
 Thorotrast-Labeled Hamster Macrophages for Various Time
 Periods in Vitro*

Days after infection	Labeled parasitophorous vacuoles
	%
1/24	52
1/6	80
1	61
3	58
7	66

that the survival mechanism of amastigotes is based on the refractory property of their surface to lysosomal degradation rather than their release of enzyme inhibitors. *L. donovani* thus resembles *Mycobacterium lepraemurium*, which lives in phagolysosomes (24), but differs from other intracellular agents, which either reside in separate vacuoles (16, 25, 26, and see 27 for other examples) or escape vacuolation by lying freely in the cytoplasm (19, 28) to avoid exposure to lysosomal enzymes. How the envelopes of leishmania amastigotes might resist the lysosomal enzyme degradation is a question that awaits further investigation. Worthy of consideration may be the possible similarity of the glycoproteins which cover the exposed surface of the parasites in phagolysosomes (14) to those which bind leukocytic lysosomal hydrolases, thereby inhibiting their activities (29).

Reorientation of amastigotes in parasitophorous vacuoles apparently occurs after incubation for longer than 1 day in vitro, resulting in the appearance of three types of vacuoles seen by transmission electron microscopy. The distended vacuolar types are not the products of fixation artifact, as they have been observed also in specimens prepared in vivo and in vitro by different methods (17, 18, and 30). It is uncertain, however, whether there actually exist 3 different types of parasitophorous vacuoles or only one type giving rise to the variations observed. Type 2 vacuoles (amastigotes partially abutting the vacuolar membrane) may conceivably give the image of type 1 (amastigotes in tight-fitting vacuoles) or type 3 (amastigotes in loose vacuoles) when sectioned at different planes. This possibility, although can be more conclusively determined by three dimensional reconstruction of parasitophorous vacuoles from serial sections, is indicated by the occurrence of forms intermediate between type 1 and 2, suggesting that the latter may be the predominant or even the only type present. It is not clearly understood why amastigotes form partial abutment against the inner lining of parasitophorous vacuoles. This phenomenon may, however, restrict the distribution of thorotrast granules to more dilated portion of these vacuoles, thereby reducing the chance of their visualization in thin sections. This may account at least in part for the reduction of labeled parasitophorous vacuoles during the postinfection periods. Evidence indicates that such large vacuoles are formed by incorporation of smaller vacuoles reminiscent of sucrosomes and the like, which are produced by repeated fusion of primary and/or secondary lysosomes with phagosomes containing nondegradable materials (31).

Our earlier finding that amastigotes not only survive but multiply in the secondary lysosomes (12) is strengthened by more cogent evidence presented for the appearance of dividing forms in labeled vacuoles coinciding with a quantitative increase of parasite numbers during the postinfection period. More significantly, evidence, not offered previously, is presented for the accumulation of extraneous dense marker in parasitophorous vacuoles, indicating that these vacuoles and the secondary lysosomes are the same. The significance of this finding merits further discussion offered below. Apparently, the parasitophorous vacuoles of leishmania constitute a continuing part of phagosome-lysosome vacuolar apparatus open to the extracellular medium, from which exogenous substances can be brought into contact with amastigotes via pinocytosis and/or phagocytosis of macrophages during the entire period of infection. These vacuoles are thus different from those of *Toxoplasma gondii*, which are intracellular "privileged" sites (32) isolated from the external milieu by a double barrier of plasma and vacuolar membranes of the host cells. Conceivably, leishmania may be more accessible than toxoplasma to the external influence by factors, such as nutrients, immunologic and chemotherapeutic agents present in the vicinity of the host cells. The ingestion of thorotrast granules into amastigotes in phagolysosome tends to confirm the validity of this suggestion. Some possible implications of this concept in several fundamental and practical aspects of intracellular parasitism of leishmaniasis can be inferred: (a) the chemical composition of ambient environment may play a more direct role than hitherto realized in the nutrition of amastigotes, although they are obligate intracellular parasites; (b) it is more possible now that lymphokines produced by sensitized lymphocytes may be transported into parasitophorous vacuoles to effect intracellular parasite destruction, for which emphasis has been placed so far on the activation of macrophages (33); and (c) a more effective chemotherapy of leishmaniasis may be achieved by the use of drugs incorporated in particulate carriers, e.g. liposomes (34, 35), which can be selectively ingested by highly phagocytic macrophages and delivered into secondary lysosomes where the parasites exist and grow. These possibilities are worthy of further investigation by means of the in vitro culture system introduced in this report.

The present in vitro culture system supports the growth of *L. donovani* amastigotes, providing a higher yield and a greater degree of reproducibility than previously obtained with the same host-parasite model (7). The growth rate obtained in vitro is nevertheless lower than that observed in vivo (36). Since the growth rate of amastigotes remains unchanged in unelicited macrophages or in the hydroxyurea-free medium, in which fibroblasts are abundant, the relatively slow growth of amastigotes in vitro cannot be accounted for by the following possibilities: (a) nonspecific activation of macrophages by stimulation of peritoneal cavity, which may increase the activities of lysosomal enzymes and thus microbicidal potential (37); (b) the presence of hydroxyurea in the culture medium; and (c) the lack of fibroblasts, which may serve the function of "nurse cells" for the parasites (7). Depletion of the essential growth factors coming with the inocula is also improbable, as amastigotes can be subcultured in vitro for at least five times in freshly isolated macrophages without significant reduction of their growth rate (38). The intrinsic differences

among macrophage subpopulations in their ability to support the growth of amastigotes may be considered as a possibility, which is noted in the present study among hamster peritoneal cells and indicated by the preliminary findings that the parasites grow more vigorously in adherent cells from bone marrow (D. M. Dwyer, unpublished observations) and blood (K-P. Chang, unpublished observations) using the same culture method (12). It deserves further investigation to determine the inherent properties of these cells in relation to their ability to support a better growth of *L. donovani* amastigotes.

Summary

An in vitro system was developed for studying host-parasite cellular interactions in visceral leishmaniasis with amastigotes isolated from infected spleens of hamsters and their peritoneal macrophages maintained by an improved method. The culture system supports the growth of *Leishmania donovani* amastigotes with different parasite/macrophage ratios for up to 2 wk, yielding results more consistent and reproducible than previously possible. Results indicated that the "forms" of the amastigotes (with or without adherent host membranes) and the "state" of the macrophages (with or without stimulation in vivo by thioglycollate or in vitro by aging) had no effect on the growth rate of the parasites, which, however, seems to vary with the macrophage subpopulations. An electron microscope study suggests that amastigotes are ingested through phagocytosis by the macrophages and become lodged in loose phagosomes. Additional evidence with quantitative data is presented to support the earlier findings that phagosome-lysosome fusion occurs after the interiorization of the parasites and that they not only survive but multiply in these vacuoles. During the postinfection periods, reorientation of amastigotes in vacuolar space results in the appearance of three types of parasitophorous vacuoles (parasites in loose vacuoles, in tight-fitting vacuoles or abutting in part against the inner lining of vacuoles). The last category may be the predominant type giving rise to the variations observed. Exogenously introduced dense marker accumulated in these parasitophorous vacuoles of the macrophages infected for several days indicating a continuous accessibility of amastigotes to the ambient medium via phagosome-lysosome vacuolar system of the host cells. This finding may have significant implications in parasite nutrition, host immunity, and chemotherapy of leishmaniasis.

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