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INTERACTION OF AVIRULENT *LEISHMANIA* SPECIES WITH RAT PERITONEAL MACROPHAGES

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An "in vitro" system has been developed for study of host cell-parasite interaction in visceral and cutaneous leishmaniasis. Avirulent promastigotes of L. brasiliensis *and* L. donovani, *from strains originally isolated from human cases and maintained by serial culture in Davis' Medium were allowed to infect cultured macrophages from rat peritoneal exudate.*

Challenge of the macrophages by parasites took place in 199 medium, at 33°C for L. brasiliensis *and at 37°C for* L. donovani. *Although the rat is resistant to infections by* Leishmania *spp., the promastigotes not only invaded the host cells, but transformed into amastigotes and later multiplied, from 10 min after challenge to 24 hours later.*

The protozoan flagellates of the genus *Leishmania* are responsible for human leishamaniasis, which can be separated clinically into the mucocutaneous and the visceral types (Garnham & Humphrey, 1969). Both types of disease, no matter what species of *Leishmania* is the pathogen, have an insect vector, a phlebotomine (Lewis, 1964), within whose alimentary tract the parasite is found outside the cells in the form of promastigotes. These forms are also found when the parasites are cultivated in cell-free media at 27°C. In the mammalian host the parasites are located within the granulocytes of the reticulo-endothelial system in the form of immobile amastigotes.

It has recently been shown that the amastigotes of *Leishmania* not only survive, but multiply in the phagolysosomal vacuoles of macrophages (Alexander & Vickerman, 1975; Chang & Dwyer, 1976; Lewis & Peters, 1977).

The present work reports on the phenomena of interiorization of avirulent promastigotes of *Leishmania brasiliensis* and *L. donovani* into the phagolysosomal vacuoles in the peritoneal exudate of white rats, as well as the transformation of the invading parasites into amastigotes, and their survival and later multiplication.

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MATERIAL AND METHODS

Parasites: Promastigotes of *L. brasiliensis* and *L. donovani* from strains maintained for 14 years in Davis' Medium with serial passage every 15 days were used in all experiments. The strains had been isolated from human cases, but had lost the capacity to produce lesion in hamsters and mice.

Harvest of macrophages and their culture: Cells from the peritoneal exudate of white rats were obtained by Sutter's Technic (1953), seeded into Leighton tubes with cover slips, and incubated 1 ½ hr at 37°C with 5% CO_2 ; 250.000 macrophages/ml were put into 2 ml 199 Medium plus 10% fetal calf serum 100 μg/ml streptomycin and 100 U/ml of penicillin.

Infection of macrophages: Promastigotes of each species were counted in a haemocytometer and added to the macrophage cultures in the ratio of 2-3 parasites/ macrophage. The preparations were then incubated at 33°C for *L. brasiliensis* and 37°C for *L. donovani.*

Estimation of intracellular infection: At intervals of 5, 10, 15, 30, and 45 minutes and 1, 2, 3, 12, 15, 18, 24, and 48 hours, duplicate cover slips were removed from each species preparation, fixed "in situ" with methanol and stained with May-Grünwald-Giemsa.

The average number of parasites inside host cells was determined for 20 fields chosen at random in areas of greatest density on each cover slip.

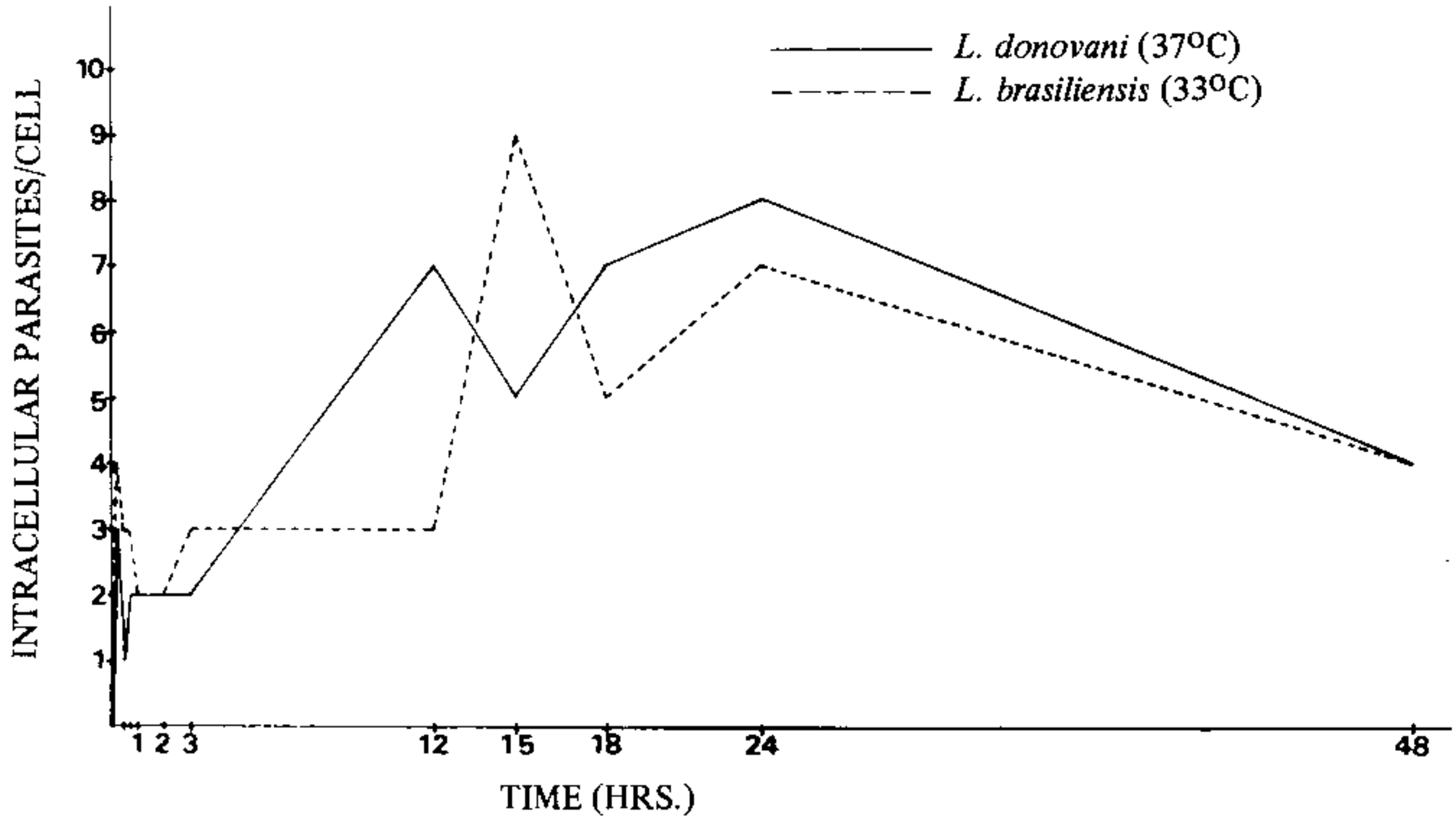
RESULTS

Light microscopy shows rapid invasion of cultured cells by both *L. brasiliensis* and *L. donovani.*

At 5 min no intracellular forms were seen, although promastigotes were seen adhering to the cover slip. Intracellular parasites began to be observed at 10 min (Fig. 1 a, b). Between 15 min and 1 hr there was an increase in parasites/macrophages (Fig. 1 c, d, e, f) and the number of parasitized cells increased (Graph 1). At the same time, flagellar regression, shortening of the parasite body, and the beginning of division were seen, **notably amastigotes with double** kinetoplasts and nuclei **(Fig. 1 g).**

GRAPH 1

Behavior of avirulent strains of *Leishmania* spp.



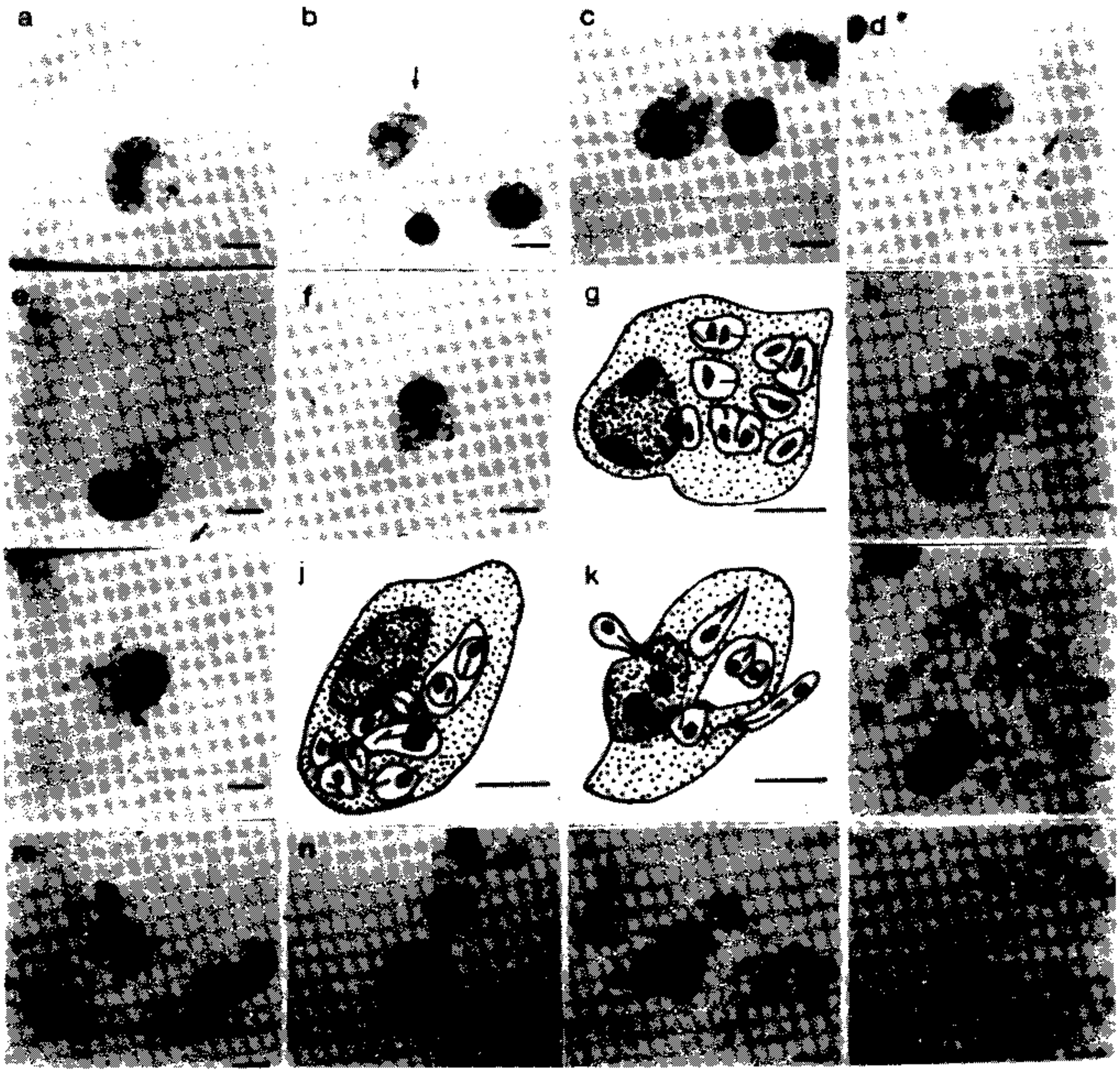


Fig. 1 – Intracellular parasites 10 min after challenge a: ***Leishmania brasiliensis***; b: *L. donovani*. – Increasing intracellular parasites, 15 min to 1 hr.; c, e: *L. brasiliensis*; d, f: *L. donovani* – Beginning of amastigote's division, note double kinetoplasts and nuclei; g: *L. brasiliensis*. (1 hr). – High multiplication speed rate 2 hours challenge; h: *L. brasiliensis*; i: *L. donovani* – Larger proportion of *L. donovani* invading host cells than *L. brasiliensis* promastigotes. Note larger size of amastigotes; j, k: *L. donovani* (2 hr). – Increasing multiplication rate and decreasing amastigote size; l, n: *L. brasiliensis* (3 and 18 hr). – Macrophage deterioration and adhesion of parasites to glass; p: *L. brasiliensis*. (48 hr).

After 2 hr, it was evident that *L. brasiliensis* was multiplying more rapidly than *L. donovani* (Fig. 1 h, i) although more of the latter had invaded host cells (Fig. 1 j, k). It was also seen that the amastigotes were larger (6-7 μ) than before.

Between 3 and 24 hr, both species continued multiplication at an increasing rate, despite similar fluctuations for both species. The amastigotes were now smaller (3-4 μ) (Fig. 1 l, m, n, o).

At 48 hr, the macrophages had deteriorated and most were lysed. Parasites were found only adhering to the cover slip (Fig. 1 p).

Table I shows the number of intracellular forms for both species. The difference in growth rate between the species is not significant.

TABLE I

Number of intracellular forms of *L. brasiliensis** and *L. donovani** in cells cultured from rat peritoneal exudate

Time	*Intracellular forms/host cell***	
	L. brasiliensis (33°C)	*L. donovani (37°C)*
5 min	0	0
10 min	1	2
15 min	4	3
30 min	3	1
45 min	3	2
1 hr	2	2
2 hr	2	2
3 hr	3	2
12 hr	3	7
15 hr	9	5
18 hr	5	7
24 hr	7	8
48 hr	4	4

* Avirulent strain
** Counts from 20 fields

DISCUSSION

Many recent studies have been made on the multiplication of different species of *Leishmania*, particularly by *L. brasiliensis* and *L. donovani* in cell cultures (macrophages and fibroblasts).

Akiyama & Taylor (1970) studied the development of promastigotes and amastigotes of *L. donovani* in hamster macrophages. Later workers have reported interiorization of various species of *Leishmania* promastigotes into cells not typically phagocytic (Akiyama & McQuillen, 1972; Lewis, 1974; Chang, 1978).

Most workers have used mouse macrophages for "in vitro" cultivation of *Leishmania*, from normal or immune mice; others have used hamster macrophages, but positive results with rat macrophages have not hitherto been reported.

Certain authors have studied the variation in susceptibility of different strains of macrophages to various *Leishmania* species. Brazil (1979) reports "in vitro" susceptibility of 2 types of mouse peritoneal macrophages to 5 *Leishmania* species, noting that only *L. aethiopica* showed notable differences to the 2 types of macrophages, and suggesting that mouse strain NHRI should be experimentally used for this species, which has been reported as being unable to infect white mice (Bray, Ashford & Bray, 1973).

Pulvertaft & Hoyle (1960) observed a reciprocal attraction between promastigotes of *L. donovani* and hamster peritoneal macrophages, the parasites adhering to the cells by the posterior extremity. Miller & Twohy (1967) saw promastigotes of the same species attaching at the tip of the flagellum, which was then engulfed by pseudopod, giving the appearance of phagocytosis.

Akiyama & Haight (1971) attempted to clarify these apparently contradictory findings, observing both types of invasion, but largely confirming the findings of Pulvertaft & Hoyle. Our own observations at 2 hr confirm the finding of Miller & Twohy (1967), although we donot discount phagocytosis. We believe that these differences may be due to experimental conditions, pH, differences in the parasite cell surfaces, variations in virulence, or differences in the macrophage strains.

Although the rat is resistant to *L. brasiliensis* infection, we have seen invasion and multiplication within 15 minutes of challenging peritoneal macrophages with this species; it is considered that penetration and phagocytosis are more active with macrophages isolated from resistant animals than from susceptible animals (Abakarova & Akinshina, 1975).

Chang (1978) considers that, whether or not the promastigotes of *Leishmania* actively penetrate host cells, their entry depends greatly on the type of cell parasitized, as well as on the species or strain of the parasite.

It is not possible to generalize on the penetrative capacity or the mechanisms of the various species of *Leishmania* into cultured cells, but it appears certain that it is a dynamic process to which both parasite and host cell contribute.

According to Lewis & Peters (1977), there are large differences between the infection of normal mouse peritoneal macrophages by promastigote forms and amastigote forms of *L. mexicana.* Promastigote infection produces small parasitophorous vacuoles that develop slowly, while amastigote infection gives large, rapidly developing vacuoles distended by fluid accumulation around the parasite. These authors, working with *L. m. amazonensis, L. m. mexicana, L. b. brasiliensis* and *L. enriettii,* observe that survival of the parasite after endocytosis by normal macrophages depends on rapid transformation to the amastigote form. They propose that the parasites survive the lysosomal enzymes in the amastigote form, whose development is related to vacuolar distension.

We have observed formation of small parasitophorous vacuoles, and often it was not possible to observe them in the first samples taken. The differentiation of promastigotes of both species studied was rapid (15 min post-infection) with no appreciable difference between the 2 species in time of development. This is in contrast to the finding of Lewis & Peters (1977), who reported that *L. enriettii, L. b. brasiliensis* and *L. m. amazonensis* transformed less rapidly into amastigotes than *L. m. mexicana,* suggestin that the speed of transformation from promastigote to amastigote is closely related to the degree of virulence. Our findings on 2 species of Leishmania, with strains totally avirulent to susceptible laboratory animal, appear to contradict this, since amastigote transformation was extremely rapid in rat peritoneal macrophages.

The effect of the lysosomal enzymes of the host cell on survival of invading *Leishmania* must be taken into account. It is well known that the parasite can survive and multiply in the phagosome-lysosome complex of the macrophage, since they are not digestible by the lysosomal enzymes, nor can they impede fusion of lysosomes in any way (Chang & Dwyer, 1976). Even though residues may be seen within parasitophorous vacuoles, derived either from the host cell or from the parasite's metabolism, the amastigotes of Leishmania can maintain their cellular integrity against the action of the lysosomal enzymes.

RESUMO

Um sistema "in vivo" foi desenvolvido para estudar-se o comportamento do parasito-célula hospedeiro em leshimaniose cutânea e visceral com promastigotos avirulentos de *L. brasiliensis* e *L. donovani* (mantidos no meio Davis) e com macrófagos de exsudado peritonial de rato. As espécies inicialmente foram isoladas de casos humanos.

A confrontação de *Leishmania* spp-macrófago se realizou na presença do meio 199 e a duas temperaturas diferentes, para *L. brasiliensis* 33°C e para *L. donovani* 37°C.

Apesar de o rato ser um animal resistente à infecção de *Leishmania* spp.; promastigotos das espécies por nós estudadas não só se interiorizaram mas também se diferenciaram em amastigotos com posterior multiplicação, a partir dos 10 minutos depois da infecção dos macrófagos e até as 24 horas.

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