Temperature-induced in vitro transformation of "Leishmania mexicana". Part I, Ultrastructural comparison of culture-transformed and intracellular amastigotes

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Objekttyp: **Article**

Zeitschrift: **Acta Tropica**

Band (Jahr): **39 (1982)**

Heft 2

Acta Tropica *39*, 143–150 (1982)

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Temperature-induced in vitro transformation of *Leishmania mexicana*

I. Ultrastructural comparison of culture-transformed and intracellular amastigotes

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Summary

Promastigotes of *Leishmania mexicana* growing exponentially in vitro at 26° C in mammalian cell culture medium with 10% serum were transformed to amastigote-like forms by elevating the incubation temperature to 34° C. During the first 24–48 h of the transformation process the slender flagellated promastigotes lost their flagella and became smaller and more ovoid. By 72 h, transformation was complete with greater than 95% of the parasites viable as judged by vital dye exclusion. Moreover, these newly transformed parasites were capable of infecting the macrophage-like tumor cell line P388D1, and could reconvert to the promastigote stage upon return to an incubation temperature of 26° C. The transformed amastigote-like parasites survived only 3 days in culture even with daily renewal of the culture medium, possibly indicating the requirement for some factor or factors normally present in the intracellular milieu. Ultrastructural studies revealed the same gross changes observed under light microscopy, and also demonstrated a close similarity in cellular organelles and membrane structure between amastigotes within phagolysosomes of P388D1 and the culture-derived amastigote forms. By these biologic and morphologic criteria the transformed parasites appear to be amastigotes, though further biochemical studies are needed to confirm their strict analogy to intracellular forms. However, the temperature-induced transformation process itself should provide an excellent model system for studying the effects of environment on eukaryotic gene expression.

Key words: *Leishmania mexicana;* amastigotes; promastigotes; transformation; electron microscopy.

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Introduction

Protozoan parasites of the genus *Leishmania* have a life cycle with two distinct developmental forms. The promastigote is an elongate flagellated form found free in the alimentary tract of vector sandflies, while in the mammalian host the smaller, ovoid, nonflagellate amastigote form is an obligate intracellular parasite of reticuloendothelial cells. In the normal course of events the promastigote forms are inoculated by sandfly bite into the mammalian host where they rapidly penetrate susceptible cells and undergo an intracellular transformation to the amastigote form (Chang, 1978). Though successful transformation is known to be crucial for survival and asexual reproduction (Lewis and Peters, 1977), little is known of the regulation of this process.

Promastigotes of many mammalian species are easily maintained in vitro in semidefined (Berens et al., 1976; Hendricks et al., 1978) or defined (Steiger and Steiger, 1977) culture media, whereas axenic amastigote culture has proven to be more difficult. Culture conditions necessary for the in vitro transformation of leishmanial promastigotes to amastigotes and the maintenance of amastigotes in culture have been reported by Lemma and Schiller (1964). However, it has been suggested that leishmanial parasites can grow at elevated temperatures as promastigotes without flagella (Trager, 1953), thus raising the possibility that the amastigotes of Lemma and Schiller were modified promastigotes. Moreover, no evidence was presented as to the morphological and biochemical analogies between culture-derived and intracellular amastigotes. Since methods for isolating viable intracellular amastigotes are difficult, the culture-derived amastigote would be a valuable asset for the study of various aspects of parasite metabolism. In this paper we describe modified in vitro culture conditions that support a solely temperature-induced transformation of *Leishmania mexicana* promastigotes to amastigotes, and transient in vitro survival of these amastigotes. Furthermore, we provide ultrastructural evidence that these amastigotes are not promastigotes without flagella and demonstrate a close similarity between these culture-derived forms and intracellular amastigotes.

Materials and Methods

Parasites. A human isolate of *Leishmania mexicana* (WR-201) was kindly provided by L. D. Hendricks, Walter Reed Army Institute of Research, Washington, D.C.

In vitro culture. Long term culture of promastigotes and transformation to amastigotes was accomplished in medium 199 (Grand Island Biological Company, Grand Islands, NY) supplemented with 1 mM sodium pyruvate, 1 mM nonessential amino acids, 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. Mishell-Dutton-approved fetal bovine serum (FBS, MA Bioproducts, Walkersville, MD) or "Hyclone" FBS from Sterile Systems, Inc., Logan, UT, was added to make a final serum concentration of 10%.

Parasite counts, measurements and viability testing. Culture flasks were vigorously mixed to insure a uniform suspension of parasites then aliquots were removed and counted using a hemacytometer in the presence of 0.2% Trypan Blue dye. Parasites which excluded the vital dye were deemed viable. For some studies, parasites were dried on microscope slides, fixed with absolute

methanol, and stained with 10% Giemsa in 0.1 M sodium phosphate buffer, pH 7.4. The stained parasites were measured under oil immersion using a calibrated ocular micrometer.

Macrophage cultures. Monolayer cultures of the macrophage-like murine tumor cell line P388D1, previously shown to be suitable hosts for *L. donovani* (Berens and Marr, 1979), were routinely maintained RPMI 1640 with 20% FBS and antibiotics in T-Flasks (25 cm^2, Costar, Cambridge, MA) with monolayer cultures were inoculated with 1.0×10^8 promastigotes, and after 72 h a significant proportion of the macrophages had intracellular amastigotes. These infected cells were removed and further processed for electron microscopy. In other studies, the ability of culture-transformed amastigotes to infect P388D1 cells was examined by adding the amastigotes to monolayer cultures in a ratio of 100:1. After 48 h, the cultures were washed, fixed with methanol, and Giemsa-stained.

Electron microscopy. Free parasites or parasitized macrophages were washed once in 0.1 M cacodylate buffer then centrifuged at $100 \times g$ for 15 min. The pellets were fixed in 2.0% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4° C. After bringing them to room temperature, the pellets were washed twice in buffer and post-fixed in 1.0% osmium tetroxide in 0.1 M cacodylate buffer for 30–45 min at room temperature. Each pellet was then washed twice in distilled water, dehydrated in a graded ethanol series, and held in propylene oxide for 30 min, then incubated in 1:1 propylene oxide-Epon overnight at 4° C. The pellets were embedded in fresh Epon, sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, then examined with a Phillips 400 or Zeiss Em 10A transmission electron microscope.

Results

Promastigotes cultured in vitro at 26° C exhibited a 24 h doubling time in the supplemented medium 199. When flasks with these actively growing promastigotes were transferred to a 34° C incubator, a process of transformation from the invertebrate to the vertebrate form was seen. The elongate (>8 μ) flagellated, motile promastigotes became nonmotile and most had lost their flagella by 20 h. A progressive contracture in size accompanied the loss of flagella and by 72 h all parasites had a maximum diameter of 5 μ or less (Table 1). Essentially all transformed parasites were viable at this time.

Table 1. Kinetics of the temperature-induced in vitro transformation of promastigotes to amastigotes

Time in 34° C culture (h)	Maximum parasite diameter (%)*			
	>8 μ	6–8 μ	4–5 μ	<4 μ
2	98	2	0	0
4	91	9	0	0
20	9	69	22	0
24	2	64	29	5
48	0	15	71	14
72	0	0	48	52

* Measurements were made with a calibrated ocular micrometer. The data represent the percent of parasites in each size category, determined from counting at least 400 total Giemsa-stained parasites.

Table 2. Culture-transformed amastigote growth and viability

Time in 34° C culture (h)	Number of viable amastigotes $\times 10^6$**	Percent of viable amastigotes
72	11.1 (4.0)	100
96	12.3 (2.7)	97
120	13.1 (1.7)	97
144	6.1 (1.3)	46
168	1.8 (0.7)	28

* Includes the 3 days required for maximum transformation of promastigotes to amastigotes. Cultures were initiated with 1.0×10^7 viable promastigotes.

** Data represent the mean hemacytometer counts of samples from 5 flasks at each time point. Numbers in parentheses indicate the standard deviation of the mean. Viability was determined by Trypan Blue dye exclusion.

Table 3. Kinetics of the temperature-induced reconversion of in vitro transformed amastigotes to promastigotes

Time in 27° C culture (h)*	Maximum parasite diameter (%)			
	>8 μ	6–8 μ	4–5 μ	<4 μ
24	0	1	99	0
48	11	39**	48	2
72	16	47	35	2

* Reconversion was started after 72 h of 34° C culture when 100% of parasites were <5 μ.

** By 48 h, all parasites 6–8 μ or larger were flagellated and motile.

To determine the length of time that the culture-transformed amastigotes would survive in vitro, flasks were maintained at 34° C and the numbers and viability of the parasites periodically assessed. Culture-transformed amastigotes showed little increase in numbers but remained viable for 120 h (Table 2). At 144 h the number of intact amastigotes had decreased significantly and only 46% of the intact cells were viable. By 168 h the cultures were obviously dying.

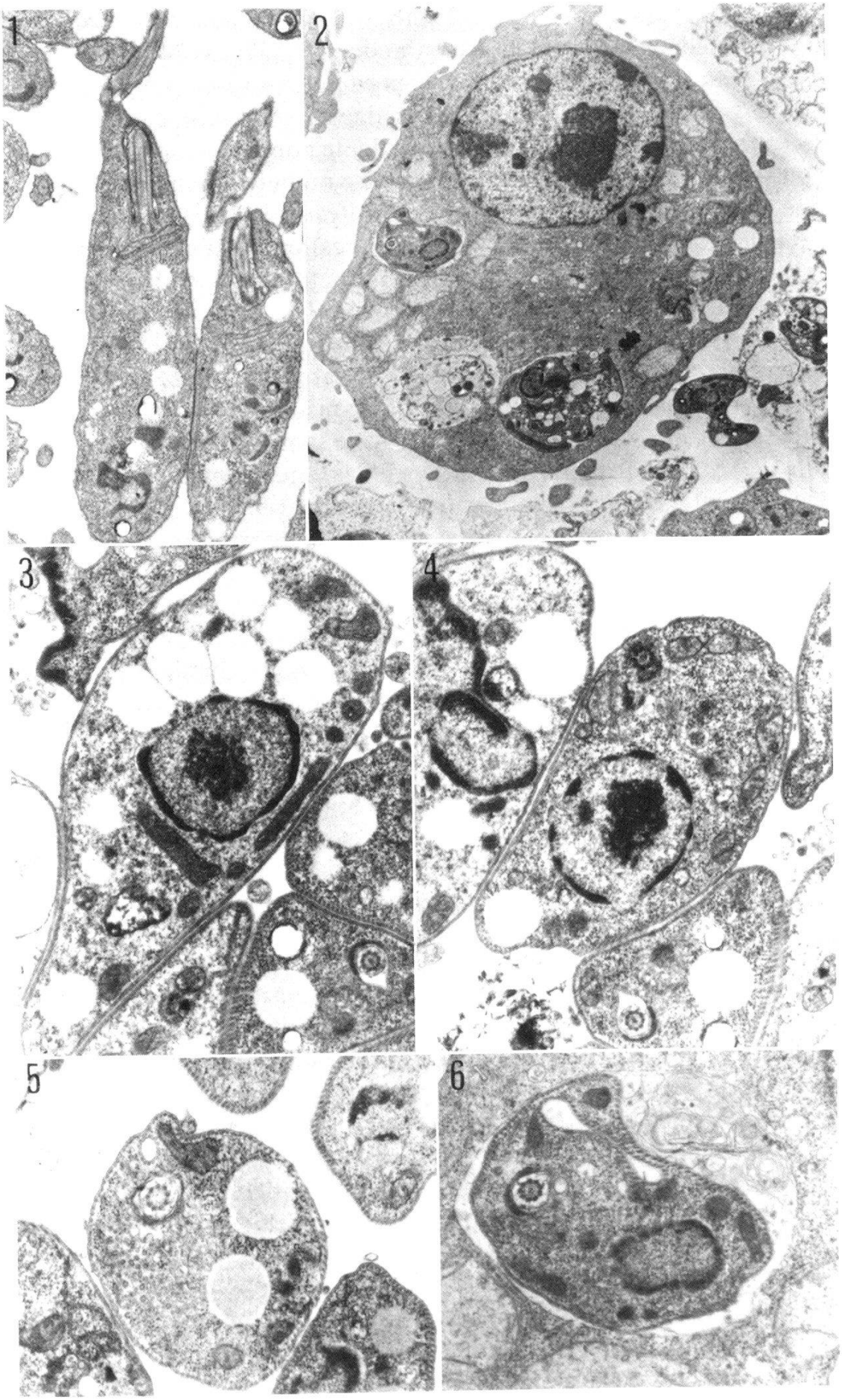
Fig. 1. *L. mexicana* promastigotes cultured at 26° C (20,000 ×).

Fig. 2. Macrophage-like tumor cell line P388D1 3 days after infection with *L. mexicana* promastigotes (7,875 ×).

Figs. 3 and 4. *L. mexicana* early transformation forms after 24 h at 34° C (31,250 ×).

Fig. 5. Transformed *L. mexicana* amastigote after 48 h in vitro at 34° C (31,250 ×).

Fig. 6. Amastigote of *L. mexicana* within phagosome of P388D1 (31,250 ×).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To further demonstrate the viability and metabolic integrity of the newly transformed amastigotes, flasks maintained for 72 h at 34° C were placed again at 26° C to see if reconversion to promastigotes would occur. The data shown in Table 3 indicate that motile promastigotes with characteristic size could be recovered from the transformed amastigote population. It was apparent that all amastigotes did not reconvert, but precise numerical estimates were impossible due to the rapid lysis of the dead parasites and the expansion of reconverted promastigote populations. Another indication of the viability of the transformed amastigotes was their ability to penetrate and establish infections within cells of the P388D1 line similar to infections by promastigotes (data not shown).

Ultrastructural studies were performed to demonstrate the morphological integrity and to assess the structural similarity of culture-transformed and intracellular amastigotes. Samples of parasites transforming in vitro were processed for electron microscopy, as were samples of the macrophage-like cell line P388D1 that contained intracellular amastigotes. Promastigotes had a characteristic elongate shape with a prominent flagellum (Fig. 1). The characteristic appearance of amastigotes within phagosomes of P388D1 cells is shown in Fig. 2. After 24 h at 34° C, the transforming promastigotes generally appeared more oval and most parasites had lost their extracellular flagella (Figs. 3 and 4). By 48 h, the parasites resembled intracellular amastigotes in size and shape (compare Figs. 5 and 6). Note the similar dimensions of the flagellar pocket and the comparable size of the cross-sectioned flagella. The culture-transformed and intracellular amastigotes possess the same intracellular organelles, most notably a mitochondrion with smaller dimensions than that seen in typical promastigotes.

Discussion

Reports of in vitro promastigote culture are numerous, but only Lemma and Schiller (1964) have reported the transformation of promastigotes to amastigotes and the subsequent maintenance of amastigote growth in biphasic culture. This early report and subsequent reports of transformation in commercial liquid media (Hendricks et al., 1978) have not documented the ultrastructural analogy of culture-derived and intracellular amastigotes. Moreover, the observation of Trager (1953) that temperature transformed amastigotes may actually be nonflagellated promastigotes cautions against the use of the term amastigote without morphologic and biochemical confirmation.

In contrast to the results of Lemma and Schiller (1964) who reported the adaptation of 3 species of *Leishmania* in in vitro culture as amastigotes by incremental increases in temperature, Gillig (1977) used a similar biphasic medium and found that *L. donovani* could be acclimatized to growth at 37° C, but as promastigotes rather than amastigotes. Lemma and Schiller (1964) also felt that the original promastigote population was variable in its genetic capaci-

ty to transform and multiply as amastigotes, and that incremental temperature increases selected for individual parasites that possessed genotypes for survival at higher temperature. Since our single step temperature change effectively transformed nearly all promastigotes to amastigotes, it appears that all individual parasites in the initial population possessed the genetic information necessary for transformation to amastigotes at 34° C.

Though the transformation of promastigotes to amastigotes appeared to be complete, the amastigotes only remained viable for 3 days despite daily renewal of culture medium. It is possible that the transformation process is strictly temperature-dependent, but survival and multiplication may require either a gradual adaptation or some as yet unknown mammalian cofactor. Studies of the transformation of newly released amastigotes to promastigotes indicate that this process is indeed temperature dependent (Akiyama and Taylor, 1970), but some observations indicate that mammalian host factors may also regulate the process (Brun et al., 1976). Products of mitogen-activated lymphocytes and homogenates from infected hamster spleens can forestall the transformation of amastigotes to promastigotes. These studies indicate a higher level of control over transformation than temperature, and provide suggestive evidence for the role of host factors in the maintenance of a particular developmental form of a parasite.

Most studies of temperature transformation in *Leishmania* have dealt with the amastigote to promastigote change. The classic ultrastructural studies of Rudzinska et al. (1964) on the transformation of *L. donovani* amastigotes to promastigotes, and the more recent report by Creemers and Jadin (1967) working with *L. mexicana* have documented the salient morphologic features of the transforming parasites. The most notable internal change was the lengthening of the mitochondrion as the amastigotes transformed into promastigotes, a process confirmed more recently by Akiyama and McQuillen (1972) who studied the ultrastructure of the reverse process in *L. donovani.* This phenomenon was also observed during culture transformation in the present study. Brun and Krassner (1976) performed a morphometric study of the mitochondrion of *L. donovani* during amastigote-promastigote transformation. They also found an increase in mitochondrial size, although the relative volume actually decreased. Another feature noted by these authors was an increased number of cristae in the kinetoplast near the flagellum. Though we did not untertake a quantitative analysis of this structural feature, in general the culture-transformed amastigotes appeared to have less cristae in this location than did the promastigotes. The most notable external changes which we observed during promastigote-amastigote transformation was the decrease in overall size from $>8\ \mu$ to $<5\ \mu$, and the rapid loss of an external flagellum. We fell that these morphologic characteristics of the culture-transformed amastigotes present sufficient dissimilarity to the promastigote form and sufficient analogy with the intracellular amastigote to call these forms true amastigotes.

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