

Ultrastructural Characterization of Serially Passaged Amastigote Like Forms of *Leishmania (Leishmania) Donovanii*

Nidhi GUPTA¹, Navdha MITTAL¹, Neena GOYAL¹,
S.C. MAITRA², and A.K. RASTOGI¹

¹Division of Biochemistry, and ²Electron Microscopy Unit, Central Drug Research Institute, Lucknow 226 001 (INDIA).

Abstract: The present study was done to establish an *in vitro* axenic culture of amastigote like forms of *Leishmania (Leishmania) donovani* (Dd-8 strain), the causative agent of Indian kala-azar. Transformation of promastigotes to amastigote like forms was induced by temperature shift from $26\pm 1^\circ\text{C}$ to $34\pm 1^\circ\text{C}$ at pH 7.0 in NNN medium. These forms were dividing as evidenced by flow cytometry. Scanning and transmission electron microscopic studies revealed a remarkable ultrastructural similarity of these *in vitro* cultured amastigotes with intracellular amastigotes. These forms have been successfully maintained for a period of more than one year, during which they have remained infective. On subjecting these forms to temperature of $26\pm 1^\circ\text{C}$, they reverted back to the promastigote forms. Thus a simple NNN medium, free from foetal calf serum has been developed to generate large amounts of amastigote like forms which can be used for further biochemical, immunological and chemotherapeutic studies.

Key words: *Leishmania (Leishmania) donovani*, amastigote like forms (ALFs), transformation, electron microscopy, NNN medium.

INTRODUCTION

Leishmania (Leishmania) donovani is the causative agent of visceral leishmaniasis (Indian kala-azar). The disease causes severe pathological, physiological and biochemical alterations in infected individuals. Symptoms of the disease include pyrexia, acute splenic and liver enlargement, hypergammaglobulinaemia and lymphadenopathy with host's immuno-suppression.

The life cycle of *Leishmania* parasite is characterized by the presence of a flagellated promastigote stage in a sandfly (Killick-Kendrick, 1979) and a non-motile amastigote stage within the phagolysosomes of macrophages in mammalian hosts (Alexander and Vickerman, 1975; Chang and Dwyer, 1976).

Received for publication, May 21, 1996.

CDRI Communication No. 5426

Correspondent author, Dr. Neena Goyal, Division of Biochemistry, Central Drug Research Institute, Lucknow 226001, India.

Promastigotes of all *Leishmania* species can be axenically cultured (Hendricks *et al.*, 1978). This has allowed extensive biological, biochemical and immunological studies. In contrast, amastigotes can be maintained in either macrophages or in animals. The purification procedures are time consuming and contamination with host material is unavoidable (Chang, 1980; Infante *et al.*, 1980; Hart *et al.*, 1981a; Handman and Curtis, 1982). Therefore, investigations on the amastigote stage, which is the pathogenic form of the parasite (Mauel, 1984), have so far been limited to morphological and some metabolic observations (Gardner *et al.*, 1977; Hart *et al.*, 1981b; Hansen *et al.*, 1984; Pimenta and DeSouza, 1988).

Several attempts have been made to culture amastigote like forms (ALFs) of different *Leishmania* species: *L. (L.) mexicana* (Pan, 1984), *L. (Viannia) panamensis* (Darling and Blum, 1987; Smejkal *et al.*, 1988), *L. (V.) braziliensis* (Stinson *et al.*, 1989; Bates, 1993). In these studies, ALFs were generated by raising the temperature of the culture medium to 34–37°C. These ALFs showed antigenic, morphological and biochemical similarities with the intracellular amastigotes (ICAs) (Smejkal *et al.*, 1988; Shapira *et al.*, 1988; Eperon and McMahon Pratt, 1989a, b; Pan *et al.*, 1993). Doyle *et al.* (1991) made continuous axenic culture of ALFs of *L. (L.) donovani* (strain Ldd 1S C12) using 100% foetal calf serum (FCS) at 37°C. The same strain was cultured in RPMI supplemented with 20% foetal calf serum at lower pH (Joshi *et al.*, 1993). Recently Castilla *et al.* (1995) have described *in vitro* culture of ALFs of another strain of *L. (L.) donovani* (LRC–L133) in TC–199 medium supplemented with 30% foetal calf serum at 38°C. However there is no report on the axenic cultivation of the causative agent of Indian kala-azar, *L. (L.) donovani* (Dd 8) amastigotes. It has been shown from our observation that *L. (L.) donovani* (Dd–8) promastigotes transform into ALFs on exposure to elevated temperature for a period of more than 48 hours (Goyal *et al.*, 1995). Recently we have characterized several membrane parameters of cultured ALFs (Gupta *et al.*, 1996). Our findings suggest a striking resemblance between the *in vitro* cultured ALFs and ICAs. In the present paper, we report a long term culture of these ALFs and their ultrastructural resemblance with ICAs.

MATERIALS AND METHODS

Parasite

Leishmania (L.) donovani (MHOM/IN/80/Dd 8) originally obtained in the form of promastigotes from Late Prof. P.C.C. Garnham, Imperial College, London (U.K.) maintained at CDRI in golden hamsters (*Mesocricetus auratus*), was used. Promastigotes were maintained in biphasic NNN (Novy, MacNeal and Nicolle) medium at 26±1°C (Chatterjee, 1981).

Intracellular amastigotes (ICAs) were isolated and partially purified from spleens of infected hamsters (Hart *et al.*, 1981a).

Transformation of promastigotes to amastigote like forms (ALFs)

Promastigotes of stationary phase culture were inoculated in fresh NNN biphasic medium (2×10^7) and transferred to 34°C ± 1 (Goyal *et al.*, 1995). The amastigote like forms

(ALFs) obtained after 96 hours of culture at 34°C were maintained by regular subcultures initially every third day and then subsequently every fifth day.

Growth kinetics

For growth kinetics, culture of ALFs was initiated with approximately 2×10^6 cells/ml inoculum and after 4, 8, 24, 30 and 48 hours of growth, cell count was determined using improved Neubauer hemocytometer.

Flow cytometry

The distribution of cells in DNA synthetic cycle of promastigotes and ALFs was analysed with Becton & Dickinson flow cytometer. Cells were fixed and stained with propidium iodide using cycle test DNA reagent kit (Vindelov *et al.*, 1983), and subjected to analysis on CELL FIT and LYSIS II software.

Infectivity

For *in vivo* infectivity, four groups of three golden hamsters each were prepared. Animals from group one and two were given intracardially an inoculum (40×10^6 cells) of promastigotes and heat stressed promastigotes (48 hours) respectively. Animals of group three and four received equal inocula of ALFs (that had been subpassaged for more than a year) and ICAs respectively (Bhatnagar *et al.*, 1989). Parasitaemia was monitored after 35 days in Giemsa stained preparations of spleens as described by Singh *et al.* (1989).

Scanning Electron Microscopy

For scanning electron microscopy (SEM), aliquots of promastigotes, ALFs and ICAs were allowed to settle onto poly-L-lysine coated glass coverslips and fixed with glutaraldehyde (3% v/v) in 0.1M cacodylate buffer (pH 7.2) for one hour at 4°C as described by Hoyer and Bucana (1982). After washing with the same buffer, cells were dehydrated in graded ethanol series (50%, 70%, 90%, 95% for 10 min each and then twice with 100% ethanol for 30 min each), followed by critical point drying in liquid CO₂ (using Balzer's Union Critical Point drier). The dried material was mounted on aluminium stubs coated with gold-palladium (Pd) alloy in a Sputter coater (Polaron E 5000) and preserved in a vacuum dessicator. Gold-Pd coated preparations were examined under Phillips SEM 515 at 25–30 kV.

Transmission Electron Microscopy

For transmission electron microscopy (TEM), promastigotes, ALFs and ICAs after harvesting and isolation, were pelleted and washed twice with phosphate buffered saline (PBS; pH, 7.2). They were fixed in glutaraldehyde (3% v/v), paraformaldehyde (2% v/v) in 0.1 M cacodylate buffer (pH, 7.2) for one hour at 4°C and then washed in the same buffer. These were then osmicated in osmium tetroxide (1% v/v in cacodylate buffer, pH 7.2). The doubly fixed samples were then washed with distilled water and embedded in 2% agar. Agar

sections were kept overnight at 4°C in 1% aqueous uranyl acetate and subsequently dehydrated in graded acetone (50%, 70%, 90%, 95% for 10 min each and then twice with absolute acetone for 30 min each). Finally the samples were embedded in a mixture of araldite and epon as described by Mollenhauer (1964). Ultrathin sections were cut on LKB-Ultramicrotome using a glass knife. Sections were stained with lead citrate and 1% aqueous solution of uranyl acetate. The stained sections were examined under Phillips 410 LS transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

Using the aforementioned protocol, we have successfully maintained the culture of ALFs for a period of more than one year involving about 105 sub-passages. Transferring the ALFs to $26 \pm 1^\circ\text{C}$ resulted in the reversion of ALFs to promastigotes with long flagella.

Figure 1 depicts typical growth curve of axenically cultured ALFs and promastigotes. No lag phase is observed in both the cases. Doubling time of ALFs is between 12–13 hours. Cultures entered stationary phase on 5th–6th day with final cell densities in the range of $3-4 \times 10^7$ cells/ml. Stationary phase persisted for about 2–3 days. After day 8–9 cell numbers began to fall, unless cultures were supplemented with fresh medium or sub-cultured. Usually the ALFs formed clumps on reaching high cell densities.

Further, two more media; 100% FCS, and RPMI–1640 supplemented with 10% FCS were also tried for the maintenance of ALFs. Whereas 100% FCS was found to be suitable for the maintenance of axenic culture of these ALFs, RPMI–1640 supplemented with 10% FCS did not support the growth of ALFs. Stationary phase in 100% FCS was observed on day 3 but was very short lived leading to rapid cell lysis on day 4.

ALFs had approximately 31%, 41% and 27% of total cell population in G_1 , S and G_2 –M phases of cell cycle while promastigotes had 13%, 64% and 25% cells in G_1 , S and G_2 –M phases respectively at the time of collection of samples.

Results of *in vivo* infectivity in golden hamsters inoculated with heat stressed promastigotes showed a slightly higher parasite burden as compared to infectivity caused by promastigotes (7.6 ± 1.4 amastigotes/100 cell nuclei and 5.0 ± 0.4 amastigotes/100 cell nuclei) respectively, whereas a significantly higher parasitic load was observed in animals infected with cultured ALFs (19.0 ± 0.6 amastigotes/100 cell nuclei) which was comparable to infectivity of the group four animals (with ICAs) (32 ± 5.6 amastigotes/100 cell nuclei) as monitored by Giemsa stained preparations.

Figures 2–4 are the SEMs showing *L. donovani* promastigotes, ALFs and ICAs. Promastigotes are elongated (9–12 μm) with long flagella and a characteristic ridge on their surfaces (Fig. 2). ALFs are slightly larger (3.0–4.0 μm) in size than the ICAs (2.5–3.0 μm) (Figs. 3 and 4). Since the medium employed was NNN (that utilizes defibrinated rabbit's blood), few red blood cells are also visible (Fig. 3). A striking resemblance between the ALFs and ICAs was observed (Fig. 4).

Figure 5 shows the typical ultrastructure of a promastigote cultured *in vitro* at 26°C .

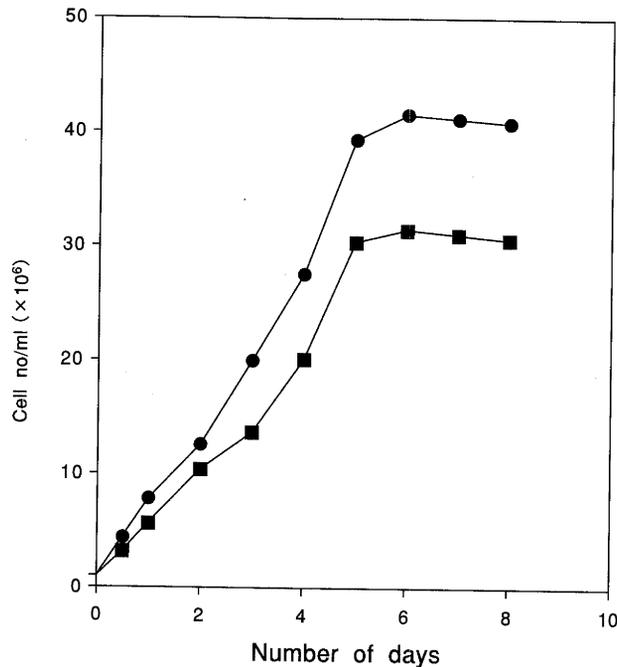
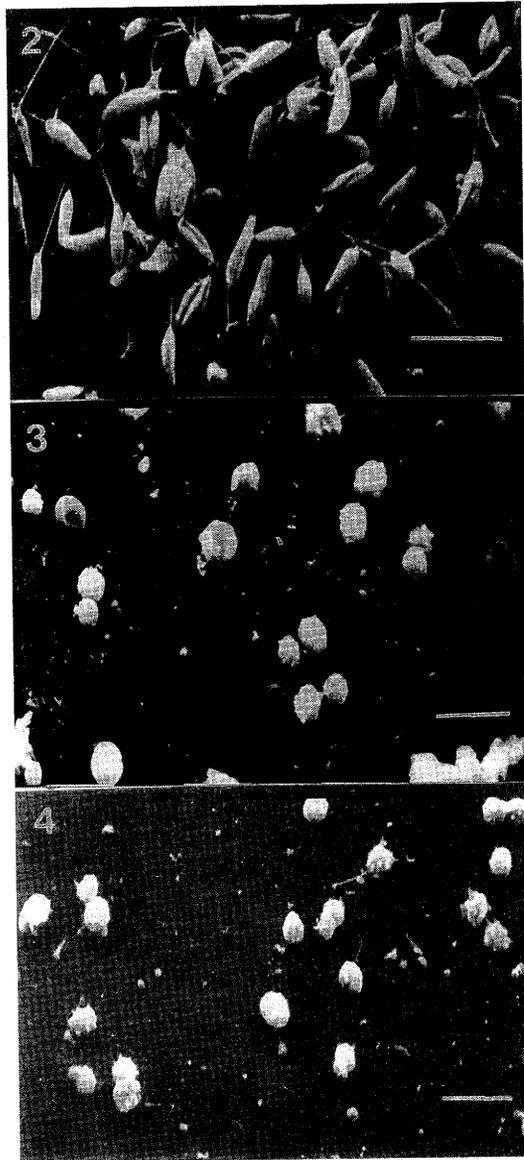


Fig 1. Growth curves of different forms of *Leishmania (L.) donovani*. Promastigotes at 26°C (●), Cultured amastigote like forms (ALFs) (■), Values are mean of three sets of experiments.

The fine structure is basically similar to that reported for other *L. (L.) donovani* strains (Rudzinska *et al.*, 1964; Brun and Krassner, 1976; Doyle *et al.*, 1991; Castilla *et al.*, 1995). Promastigotes are 8 μm in length and 2.0–2.4 μm wide with a long flagella (Chatterjee and SenGupta, 1970). Flagellum arising at the base of the flagellar pocket extends beyond the reservoir and contains a paraxial rod. The flagellar pocket is usually not distended and lacks the presence of any particular vesicle or granules. The kinetoplast is situated anterior to the nucleus, just below the base of the flagellar pocket. The kinetoplast DNA (k-DNA) occupies nearly half of the kinetoplast area. Mitochondria showing cristae are also observed, though they are small in size and few in number. The surface membrane is fine and shows subpellicular microtubules arranged in a row.

Figures 6–9 are the TEM micrographs of various stages of *in vitro* transformation of promastigotes to amastigotes at elevated temperatures. Figure 6 shows a transforming promastigote which is reduced both in cell size (4.5 μm) and length of the flagellum. Figure 7 illustrates a nearly rounded form (with a diameter of 4.3 μm) along with the remnants of a cut flagellum at the upper part. Figure 8 illustrates the finer details of conversion of promastigote to ALFs: the cell has rounded up completely, the flagellum is being shed, and the membrane around the flagellar pocket has extended so as to enclose the remaining part



Figs 2–4: Scanning micrographs of different stages of *Leishmania (L.) donovani*. 2. Promastigotes; elongated, flagellated. 3. Cultured ALFs; round, aflagellated. 4. Intracellular amastigotes (ICAs) round, aflagellated. Bar=10 μm in Figure 2; 5.0 μm in others.

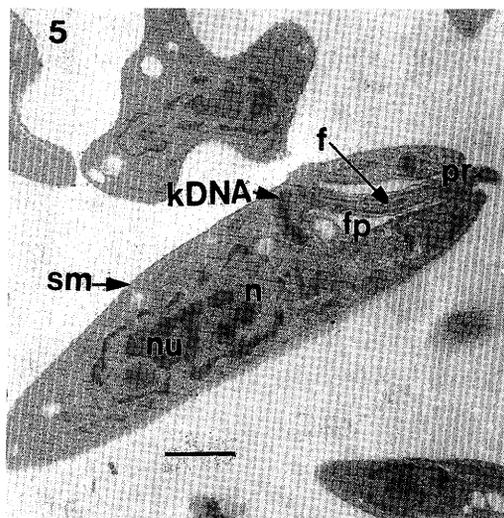
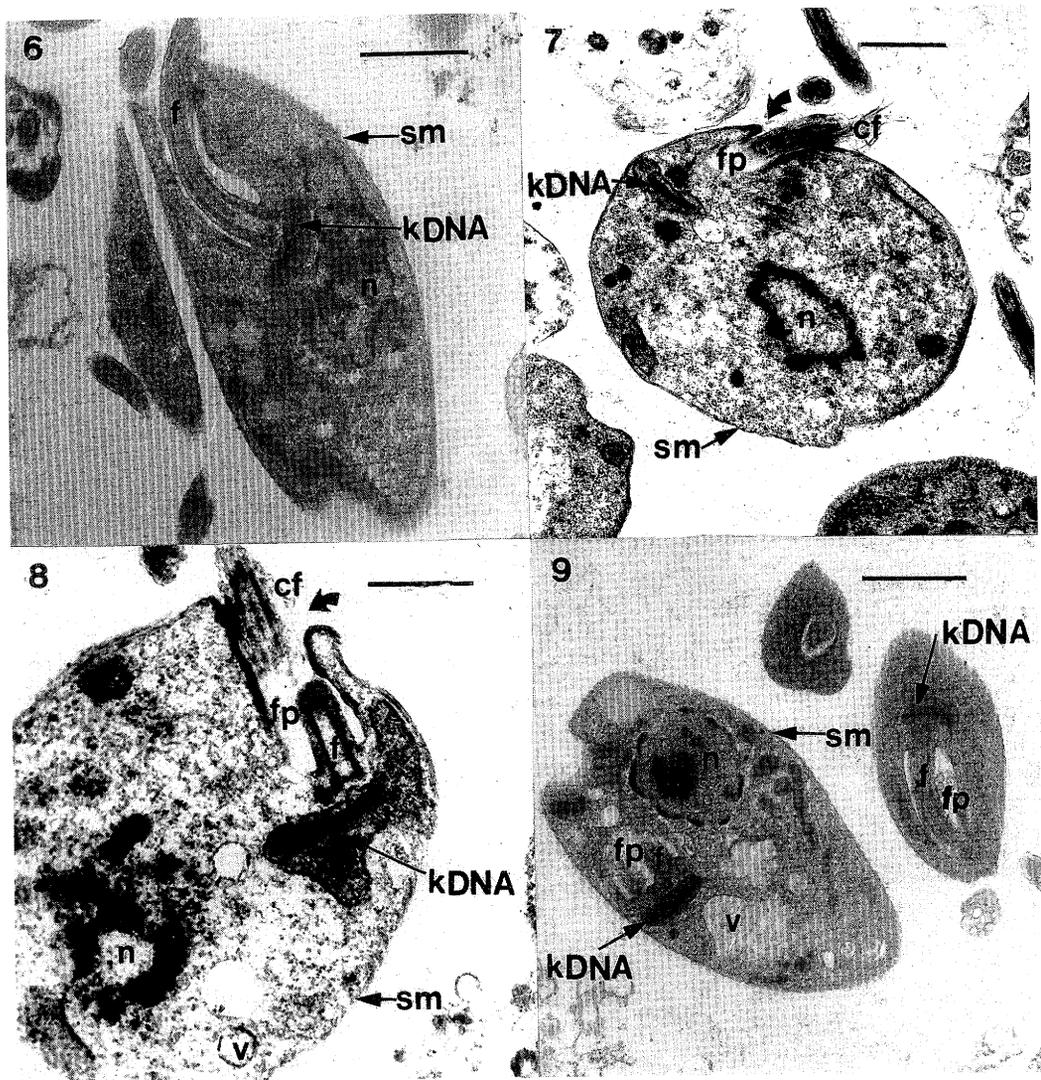


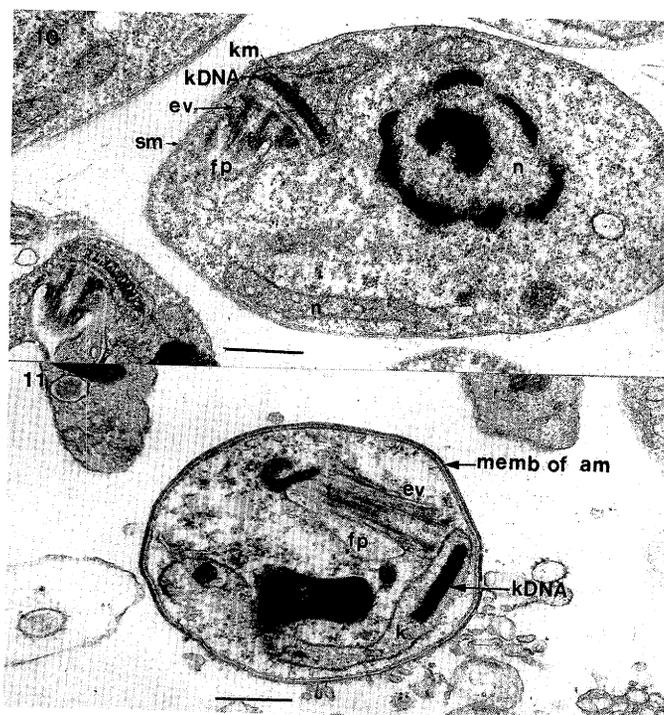
Fig 5: Transmission electron micrograph (TEM) of a promastigote of *Leishmania (L.) donovani*. Elongated with free flagellum. n, nucleus; nu, nucleolus; sm, surface membrane; kDNA, kinetoplast DNA; f, flagellum; fp, flagellar pocket; pr, paraxial rod, Bar=1 μm .

of the flagellum. Figure 9 shows completely transformed dividing amastigote like forms, the larger one has completely rounded off ($3.7 \mu\text{m}$) while the other one is further reduced in size ($2.3 \mu\text{m}$) with the flagella completely enclosed inside flagellar pockets. Rest of the cellular organelles e.g. mitochondria, ribosomes, vacuoles are also distinct. The TEM micrograph in Figure 10 shows cultured ALFs with all the ultrastructural details. The nucleus is well characterized with the nuclear pore and nucleolus. The subpellicular microtubules of the surface membrane are present in an array. The kinetoplast with the k-DNA strands is slightly enlarged. Degeneration in mitochondrial vesicles is observed, which is consistent with the previous report (Brun and Krassner, 1976). The most characteristic feature (showing similarity with ICAs) is the presence of a non-emergent flagellum. Some granules are also present at the base of the flagellum.

Figure 11 is the TEM micrograph of ICAs. These are found to be smaller than ALFs cultured *in vitro* at elevated temperatures ($2.0 \mu\text{m} \times 1.7 \mu\text{m}$). The flagellum of the amastigote remains enclosed in the flagellar pocket and lacks a paraxial rod. Also the flagellar pocket is greatly distended forming a sort of reservoir, harbouring numerous electron dense exocytotic vesicles. The cytoplasm too contains several electron dense structures.



Figs 6–9: TEMs of *Leishmania (L.) donovani* at different stages of transformation. 6. Promastigote at 34°C for 24 hr. 7. Promastigote at 34°C for 48 hr, rounded. 8. Transforming cell; flagellum shed, plasma membrane grows to enclose flagellar pocket. 9. Two transformed cells; flagella completely enclosed in flagellar pocket. n, nucleus; kDNA, kinetoplast DNA; f, flagellum; fp, flagellar pocket; cf, cut-off flagellum; sm, surface membrane; v, vacuole. Bar=1.5 μ m.



Figs 10 and 11: TEMs. 10. Cultured ALFs; flagellum completely enclosed in flagellar pocket with exocytotic vesicles. 11. ICA. n, nucleus; kDNA, kinetoplast DNA; km, kinetoplast membrane; f, flagellum; fp, flagellar pocket; sm, surface membrane; ev, exocytotic vesicles; memb of am, membrane of amastigote. Bar=0.5 μ m.

DISCUSSION

The transformation of promastigote to amastigote in *Leishmania* occurs during phagocytosis by host phagocytes. Among various factors that trigger the transformation process, pH and temperature have been shown to play key roles (Darling and Blum, 1987; Smejkal *et al.*, 1988; Stinson *et al.*, 1989; Zilberstein *et al.*, 1991). However temperature shift (26° to 34–38 °C) appears to be sufficient to achieve the *in vitro* transformation of promastigotes to ALFs of several *Leishmania* species including *L. (L.) donovani* (Pan, 1984; Smejkal *et al.*, 1988; Eperon and MacMahon–Pratt, 1989a; Doyle *et al.*, 1991; Castilla *et al.*, 1995).

So far, ALFs of only one strain of *L. (L.) donovani* i. e. Ldd C12 IS have been successfully cultured for a period of more than 6 months in various culture systems varying from 100% FCS to 20% FCS supplemented with RPMI–1640 (Doyle *et al.*, 1991; Joshi *et al.*, 1993). We report for the first time, a successful cultivation of ALFs of *L. (L.) donovani* (strain Dd 8) in a medium completely devoid of FCS, i. e. NNN medium. This is a typical medium of choice due to its relative ease and low cost as compared to the other culture media employed. We have also used 100% FCS and RPMI–1640 supplemented with 10% FCS. Only

in FCS (100%) ALFs multiply, but there is no stationary phase and cell lysis occurs very fast as observed by Doyle *et al.* (1991). In NNN medium, cell doubling time varies from 12–13 hours which is comparable to that of ALFs of strain Ldd C12 1S of *L. (L.) donovani* (Doyle *et al.*, 1991).

The most obvious morphological changes that occur during transformation are in the shape, size and length of the cell body. SEMs reveal a drastic decrease in the length of cells, followed by loss of flagella and rounding of cells (Figs. 2–4). These ALFs are viable, dividing and infective. The infectivity of serially passaged *L. (L.) donovani* ALFs was greater than promastigotes and comparable to the infectivity caused by ICAs in golden hamsters. This is in accordance with a previous report on increased infectivity of parasites due to heat stress in *L. (Viannia) panamensis* (Smejkal *et al.*, 1988; Goyal *et al.*, 1995). Further, it is also known that intracellular amastigotes have a greater infectivity for experimental animals than the promastigotes, possibly due to their increased ability to survive the defense mechanisms of the host (Mauel, 1984).

Examination of ALFs by TEM reveals that the *in vitro* cultured ALFs are similar to amastigotes isolated from hamster spleens in ultrastructure. In the ALFs, the flagellum is confined to the flagellar pocket. The paraxial rod is absent in the flagellum and exocytotic vesicles are also observed (Fig. 10). All these features are characteristic of ICAs (Pan and Pan, 1986). Since amastigote is the non-motile form restricted to the parasitophorous vacuole of macrophages, its flagellum is confined to the flagellar pocket only and lacks a paraxial rod. Exocytotic vesicles are speculated to be secretory in nature and may be responsible for the secretion of hydrolytic/proteolytic enzymes needed for the survival of the parasite. The ALFs of *L. (L.) donovani* showed an absence of megasomes, the organelles characteristic of axenically cultured amastigotes of *L. (L.) mexicana* (Bates *et al.*, 1992).

The mitochondria (both in number and size) showed a degenerating pattern during transformation from promastigote to amastigote, which is well consistent with other previous reports on mitochondrial development during transformation (Rudzinska *et al.*, 1964; Brun and Krassner, 1976).

Thus, we conclude that NNN medium is an optimum medium to cultivate ALFs of *L. (L.) donovani in vitro* at 34°C. These ALFs are ultrastructurally similar to intracellular amastigotes and can be transformed to promastigotes simply by decreasing the culture temperature to 26°C. These ALFs can be utilized for several immunological, biochemical and chemotherapeutic studies.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. V.P. Kamboj, FNA, Director, C.D.R.I., Lucknow for providing the necessary facilities. Financial assistance in the form of Junior Research Fellowships to N. Gupta and N. Mittal from CSIR and ICMR, New Delhi respectively is gratefully acknowledged. The authors thank Dr. N.K. Ganguly Professor and Head, Department of Experimental Medicine, PGIMER, Chandigarh and Dr. Harpreet Vohra, PGIMER, Chan-

digarh for flow cytometry analysis, and Dr. P.Y. Guru, CDRI for providing microscopy facilities. Technical assistance by Mrs. Abha Arya is also gratefully acknowledged.

REFERENCES

- 1) Alexander, J. & Vickerman, K. (1975): Fusion of host cell secondary lysosomes with parasitophorous vacuoles of *L. mexicana* infected macrophages. *J. Protozool.*, 22, 502–508.
- 2) Bates, P.A. (1993): Axenic culture of *Leishmania* amastigotes. *Parasitology Today* 9, 143–146.
- 3) Bates, P.A., Robertson, C.D., Tetley, L. & Coombs, G.H. (1992): Axenic cultivation and characterization of *Leishmania mexicana* amastigote like forms. *Parasitol.*, 105, 193–202.
- 4) Bhatnagar, S.; Guru, P.Y., Katiyar, J.C., Seth, M. & Bhaduri, A.P. (1989): Exploration of antileishmanial activity in heterocycles; results of their *in vivo* and *in vitro* bioevaluations. *Ind. J. Med. Res.*, 89, 439–444.
- 5) Brun, R. & Krassner, S.M. (1976): Quantitative ultrastructural investigations of mitochondrial development in *Leishmania donovani* during transformation. *J. Protozool.*, 23, 493–497.
- 6) Castilla, J.J., Sanchez–Moreno, M., Mesa, C. & Osuna, A. (1995): *Leishmania donovani*: *In vitro* culture and [¹H] NMR characterization of amastigote like forms. *Mol. Cellul. Biochem.* 142, 89–97.
- 7) Chang, K.P. (1980): Human cutaneous leishmania in a mouse macrophage cell line: propagation and isolation of intracellular parasites. *Science* 209, 1240–1242.
- 8) Chang, K.P. & Dwyer, D.M. (1976): Multiplication of a human parasite (*L. donovani*) in phagolysosomes of hamster macrophages *in vitro*. *Science* 193, 678–680.
- 9) Chatterjee, K.D. (1981): Subphyllum Sarcomastigophora. pp54. In: Chatterjee K.D. (ed). *Parasitology*. Chatterjee Medical Publishers, Calcutta (India).
- 10) Chatterjee, S.N. & Sengupta, P.C. (1970); Ultrastructure of the promastigote of *Leishmania donovani*. *Ind. J. Med. Res.*, 58, 70–76
- 11) Darling, T.N. & Blum, J.J. (1987): *In vitro* reversible transformation of *Leishmania braziliensis panamensis* between promastigote and ellipsoidal forms. *J. Protozool.*, 34 (2), 166–168.
- 12) Doyle, P.S., Engel, J. C., Pimenta, P.F., Dasilva, P.P. & Dwyer, D.M. (1991): *Leishmania donovani*: Long–term culture of axenic amastigotes at 37°C. *Exp. Parasitol.*, 73, 326–334.
- 13) Eperon, S. & McMahon–Pratt, D. (1989a): Extracellular cultivation and morphological characterization of amastigote like forms of *Leishmania panamensis* and *L. braziliensis*. *J. Protozool.* 36, 502–510.
- 14) Eperon, S. & McMahon–Pratt, D. (1989b): Stage and species specific monoclonal antibodies. *J. Protozool.*, 36, 510–518.
- 15) Gardner, P.J., Schory, L. & Chance, M.L. (1977): Species differentiation in the genus *Leishmania* by morphometric studies with the electron microscope. *Ann. Trop. Med. Parasitol.*, 71, 147–155.
- 16) Goyal, N., Singh, A.K., Guru, P.Y. & Rastogi, A.K. (1995): Biochemical changes in heat stressed promastigotes of *L. donovani*. *Ind. J. Exp. Biol.*, 33, 222–226.
- 17) Gupta, N., Goyal, N., Kumar, R., Agarwal, A.K., Seth, P.K. & Rastogi, A.K. (1996): Membrane characterization of amastigote like forms of *Leishmania donovani*. *Trop. Med. Intn. Health.*, (In press).
- 18) Handman, E. & Curtis, J.M. (1982): *Leishmania tropica* surface antigens of intracellular and flagellate forms. *Exp. Parasitol.*, 53, 243–249.
- 19) Hansen, B.D., Webster, H.K., Hendricks, L.D. & Pappas, M.G. (1984): *Leishmania mexicana*: Purine metabolism in promastigotes, axenic amastigotes and amastigotes derived from vero cells. *Exp.*

- Parasitol., 58, 101–109.
- 20) Hart, D.T., Vickerman, K. & Coombs, G.H. (1981a): A quick, simple method for purifying *Leishmania mexicana* amastigotes in large numbers. Parasitol. 82, 345–355.
 - 21) Hart, D.T., Vickerman, K. & Coombs, G.H. (1981b): Respiration of *Leishmania mexicana* amastigotes and promastigotes. Mol. Biochem. Parasitol., 4, 39–51.
 - 22) Hendricks L.D., Wood, D.E. & Hajduk, M.E. (1978): Haemoflagellates: Commercially available liquid media for rapid cultivation. Parasitol., 76, 309–316.
 - 23) Hoyer, L.C. & Bucana, C. (1982): pp 233–271. In J.J. Marchalories & G.W. Warr (ed.) Principles of Immunoelectron Microscopy, John Wiley London.
 - 24) Infante, R.B., Hernandez, A.G., Riggione, F. & Dawidowicz, R. (1980): A new method for the purification of *Leishmania* amastigotes from cutaneous lesions. Parasitol., 80, 105–112.
 - 25) Joshi, M., Dwyer, D.M. & Nakhasi, H.L. (1993): Cloning and characterization of differentially expressed genes from *in vitro* grown amastigotes of *Leishmania donovani*. Mol. Biochem. Parasitol., 58, 345–355.
 - 26) Killick–Kendrick, R. (1979): Biology of *Leishmania*. pp. 395–460 In W.H.R. Lumsden & D.A. Evans (ed). Biology of the Kinetoplastida, Academic Press, London.
 - 27) Mauel, J. (1984): Mechanism of survival of protozoan parasites in mononuclear phagocytes. Parasitol., 88, 579–592.
 - 28) Mollenhauer, H.H. (1964): Stain Technology 39, 111.
 - 29) Pan, A.A. (1984): *Leishmania mexicana*: Serial cultivation of intracellular stages in a cell–free medium. Exp. Parasitol., 58, 72–80.
 - 30) Pan, A.A., & Pan, S.C. (1986): *Leishmania mexicana*: Comparative fine structure of amastigotes and promastigotes *in vitro* and *in vivo*. Exp. Parasitol., 62, 254–265.
 - 31) Pan, A.A., Duboise, S.M., Eperon, S., Rivas, L., Hodgkinson, V., Traubcasko, Y. & McMahon–Pratt, D. (1993): Developmental life cycle of *Leishmania*—Cultivation & characterization of cultured extracellular amastigotes. J. Euk. Microbiol. 40 (2), 213–223.
 - 32) Pimenta, P.F.P. & DeSouza, W. (1988): Freeze–fracture and cytochemistry study of the interaction between *Leishmania mexicana amazonensis* and macrophages. Journal of Submicroscopy and Cytological Pathology 20, 89–99.
 - 33) Rudzinska, M.A, D'alesandro P.A. & Trager, W. (1964): The fine structure of *Leishmania donovani* & the role of kinetoplast in the leishmania–leptomonad transformation. J. Protozool., 11, 166–191.
 - 34) Shapira, M., McEwen, J.G. & Jaffe, C.L. (1988): Temperature effects on molecular processes which lead to stage differentiation in *Leishmania*. EMBO Journal 7, 2895–2901.
 - 35) Singh, A.K., Guru P.Y., Sharma, S.K., Rastogi, A.K. & Garg, N.K. (1989): Changes in the liver and spleen of golden hamsters during *Leishmania donovani* infection. Med. Sci. Res., 17, 787–789.
 - 36) Smejkal, R.M., Wolff, R. & Olenick, J.G. (1988): *Leishmania braziliensis panamensis*: Increased infectivity resulting from heat shock. Exp. Parasitol., 65, 1–9.
 - 37) Stinson, S., Sommer, J.R. & Blum, J.J. (1989): Morphology of *Leishmania braziliensis*: Changes during reversible heat induced transformation from promastigote to an ellipsoidal form. J. Parasitol., 75, 431–440.
 - 38) Vindelov, L.L., Christensen, I.J. & Nissen, N.L. (1983): A detergent trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry 3, 323–327.
 - 39) Zilberstein, D., Blumenfeld, N., Livacru, V., Gepstein, A. & Jaffe, C.L. (1991): Growth at acidic pH induces an amastigote specific protein in *Leishmania* promastigotes. Mol. Biochem. Parasitol., 45, 175–178.