Review Article

The axenic cultivation of Leishmania donovani amastigotes

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ABSTRACT

Leishmania parasites cause a wide range of human diseases from localized self-healing cutaneous lesions to fatal visceral disease. These parasites are characterized by having a non-motile form, amastigotes, within the macrophage of the mammalian host and a motile form, promastigotes, in the sand fly. Transformation of promastigotes has been attracting considerable attention in studying the parasite-host interaction. It is the purpose of this article to review the transformation of promastigotes to amastigotes and the growth of the later form in a cell free medium, i.e. axenically. Also, results of original work on axenic amastigotes of *Leishmania donovani* are shown. The effect of temperature, pH, and gas requirements on the transformation and further growth of *Leishmania donovani* axenic amastigotes are discussed.

Keywords: *Leishmania, donovani*, amastigotes, axenic culture.

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Leishmania parasites causes a wide range of human diseases from localized self-healing cutaneous lesions to fatal visceral disease. These parasites have a life cycle characterized by the presence of flagellated promastigotes in the sandfly host and non-motile amastigotes within the phagolysosomes of macrophages in the mammalian host.

To study these parasites, promastigotes and amastigotes have been cultured under different *in vitro* laboratory conditions (reviewed by Evans[1]) and have been the subject of numerous biological and biochemical studies. Promastigotes of *Leishmania* species are usually cultured *in vitro* in culture media of neutral pH at 25-27°C. They are easily grown and most biochemical research on *Leishmania* has dealt with this form. Such cultured promastigotes are more likely to share properties with those found in infected sandflies than with amastigotes in phagolysosomes of macrophages in the mammalian host. Therefore, knowledge of the properties of promastigotes may not improve understanding of the disease itself.

This can be overcome by studying the amastigotes that are responsible for the pathology of leishmaniasis. Amastigotes are an intracellular stage in macrophages and can be obtained from infected animals or from *in vitro* cultures in macrophage-like cell lines. However, there is often a doubt regarding the purity of amastigotes derived from such sources. This can be overcome by the cultivation of amastigotes in a cell-free medium, i.e. axenically.[2] The first reliable method for cultivation of amastigote-like forms in liquid medium was that described by Pan.[3] Using a similar methodology, other workers have been able to culture a variety of other species as amastigote-like forms.[4-9]

Axenically cultured amastigotes should be proven not only to differ from promastigotes but, more importantly, to be similar to lesion amastigotes. A

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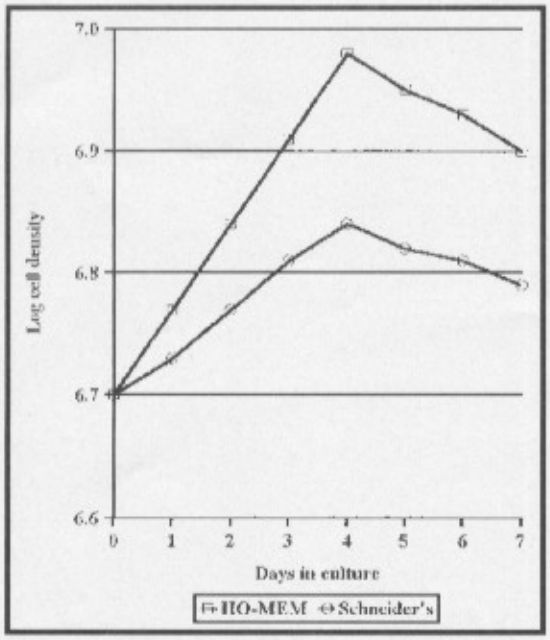

Figure 1 - Growth of *L.donovani* axenic amastigotes in HO-MEM medium and Schneider's drosophila medium both supplemented with 20% FCS.

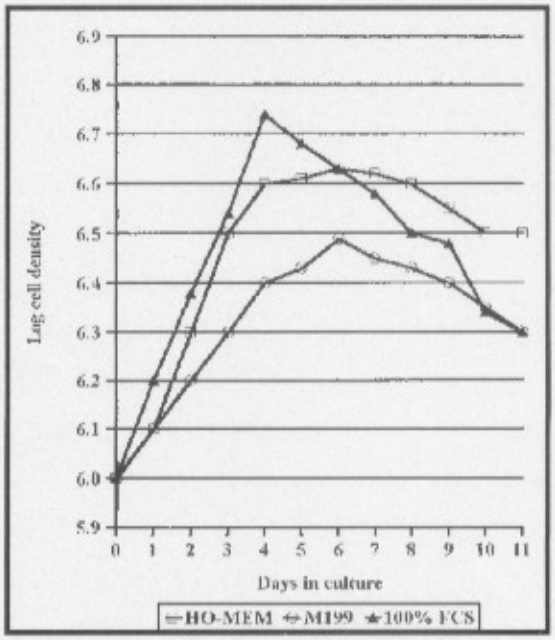

Figure 2 - Growth of *L.donovani* axenic amastigotes in HO-MEM medium, M199 both supplemented with 20% FCS and in 100% FCS.

variety of criteria can be used to study these differences and similarities.

Optimum conditions for the growth of axenic amastigotes. Several studies have recently focused on the transformation and axenic cultivation of amastigotes (reviewed by Bates[2]). Different factors that contribute to the transformation and growth of axenic amastigotes have been investigated in this study.

Culture medium. Currently, there is no single universal culture medium to grow all stages of different species of *Leishmania* and it is difficult to predict which culture medium will optimally support a specific isolate of *Leishmania* in advance.[1] In our study, 3 culture medias were used to grow axenic amastigotes of *L.donovani* (MHOM/ET/67/HU3; LV9) originally transformed in HO-MEM medium supplemented with 20% FCS at a pH of 5.5 at 37°C. These were HO-MEM medium, M199 and Schneider's *Drosophila* medium and all were supplemented with 20% FCS. FCS was also used on its own as a culture medium. Amastigotes were inoculated into 10 ml of fresh medium in 25 cm^2 tissue culture flasks. Cultures were incubated at 37°C in the presence of 5% CO_2. Cell densities were estimated every 48 hours. HO-MEM medium supplemented with 20% FCS was the best of these tested for the transformation and axenic growth of *L. donovani* amastigotes. This was judged by the higher cell densities and faster rate of growth of amastigotes grown in this medium (Figure 1 and 2).

Temperature. Temperature plays an important role in the transformation of promastigotes to amastigotes.[10] In all efforts to grow amastigotes axenically reported to date, the temperature was an important factor. In our study, the temperature for culturing axenic amastigotes was chosen to be 37°C, the temperature of the viscera of the mammalian host. Temperature was not a factor that was optimized: it was considered that growth at 37°C was a prerequisite for axenic amastigotes of *L.donovani*. Preliminary experiments revealed that gradual increase of temperature by adapting promastigotes to grow at 32°C and then 35°C, before transferring them to 37°C was not successful in obtaining axenic amastigotes whereas direct transfer to 37°C succeeded. This could be due to an adaptation to grow as promastigotes *in vitro* at 32°C and/or 35°C and subsequent loss of their ability to transform to amastigotes.

pH. Another important factor in the axenic cultivation of *Leishmania* amastigotes is the pH of the culture medium. Induction of transformation to the amastigote stage by low pH has also been reported in *Trypanosoma cruzi*.[11] A pilot study was carried out to find the optimum pH for the transformation and growth of axenic amastigotes in HO-MEM medium supplemented with 20% FCS. The culture medium was prepared at different pH values ranging from 4.0 to 6.5 and inoculated with axenic amastigotes at a density of $5x10^6$. We found that a pH of 5.5 is optimal for the transformation and subsequent growth of *L.donovani* amastigotes

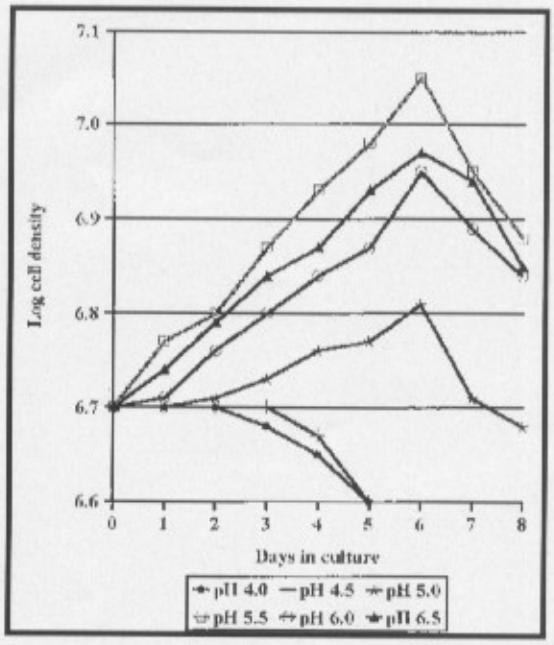

Figure 3 - Growth of *L.donovani* axenic amastigotes in HO-MEM medium supplemented with 20% FCS at different pH.

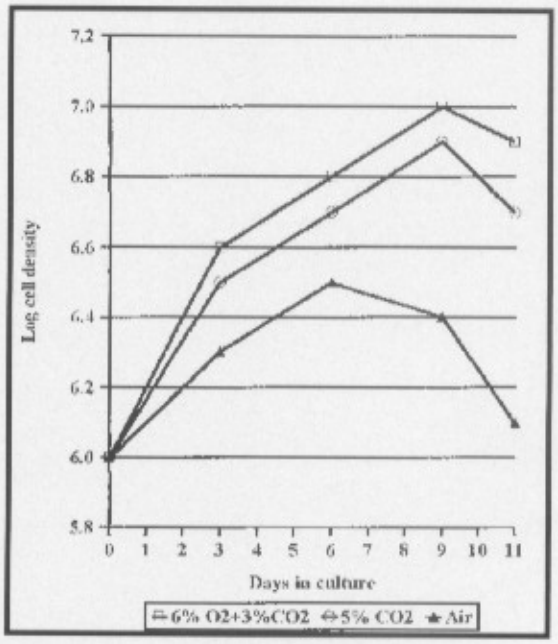

Figure 4 - Growth of *L.donovani* axenic amastigotes in different gas conditions.

axenically (Figure 3). This agrees with the findings of Bates et al[7] who were able to grow amastigotes of *L.mexicana* axenically. The parasitophorous vacuoles of *L.amazonensis*-infected macrophages were found to maintain an acidic pH of 4.7 to 5.3.[12] Although unknown, the pH of the sandfly mid gut is likely to be relatively alkaline[13] and, hence, the transformation of promastigotes to amastigotes, which occurs during phagocytosis by host macrophages, could be stimulated by a rapid exposure to an acidic environment. Amastigotes and promastigotes of *Leishmania* were shown to have developed mechanisms to sense changes in environmental pH.[10] Furthermore, although *L.donovani* promastigotes metabolize glucose, proline, and nucleosides optimally at pH 7.0-7.5, amastigotes catabolize these substrates optimally at pH 4.5-5.0.[14] Amastigote stage-specific proteins were observed among promastigotes of *L.major* when cultured in acidic pH of 4.5.[15]

Gas requirements. Gas requirements for *Leishmania* amastigote culture have not been investigated thoroughly before. Freshly transformed amastigotes were cultured in HO-MEM medium supplemented with 20% FCS pH 5.5 in different gas conditions: 100% CO_2 (anaerobic); air; 5% CO_2/air; and 6% O_2- 3% CO_2- 91% N_2. For the anaerobic conditions, cultures were incubated in an anaerobic jar. For air as the gas phase, culture flasks were closed during incubation in the 5% CO_2 incubator and opened daily in a laminar flow hood to allow gas exchange. For 5% CO_2/air gas phase, the lid of the culture flask was left slightly open inside the incubator to allow gas exchange. For the 6% O_2- 3% CO_2- 91% N_2 gas phase, a sterile stream of the gas mixture was blown inside the culture flask daily. Cultures were incubated at 37°C and the cell densities were estimated every 3 days. We found that amastigotes grew better in the presence of a high carbon dioxide concentration. A slight improvement in growth was observed when the oxygen concentration was also decreased to 6% (Figure 4). Gas conditions have also been shown to affect amastigote to promastigote transformation and promastigote metabolism. Hart and Coombs[16] reported that a high carbon dioxide concentration increased the rate of amastigote to promastigote transformation whereas lowering the oxygen tension slightly improved the rate of transformation. They concluded that amastigotes were adapted to grow in low oxygen tensions encountered *in vivo*. Keegan and Blum[17] reported that glucose consumption in *L. major* promastigotes increased as the concentration of oxygen was reduced to 6%.

Characterization of axenic amastigotes. After optimizing the technique for the axenic cultivation of *L.donovani* amastigotes, it was essential to compare the amastigote-like forms obtained from such cultures with the tissue amastigotes obtained from active infection in animals and promastigotes from *in vitro* cultures. This was performed by comparing three criteria; (1) morphology, (2) ultrastructure, and (3) protein banding pattern on SDS-PAGE.

Morphology under light microscopy. Morphologically, axenic amastigotes were very similar to tissue amastigotes in that they appeared as

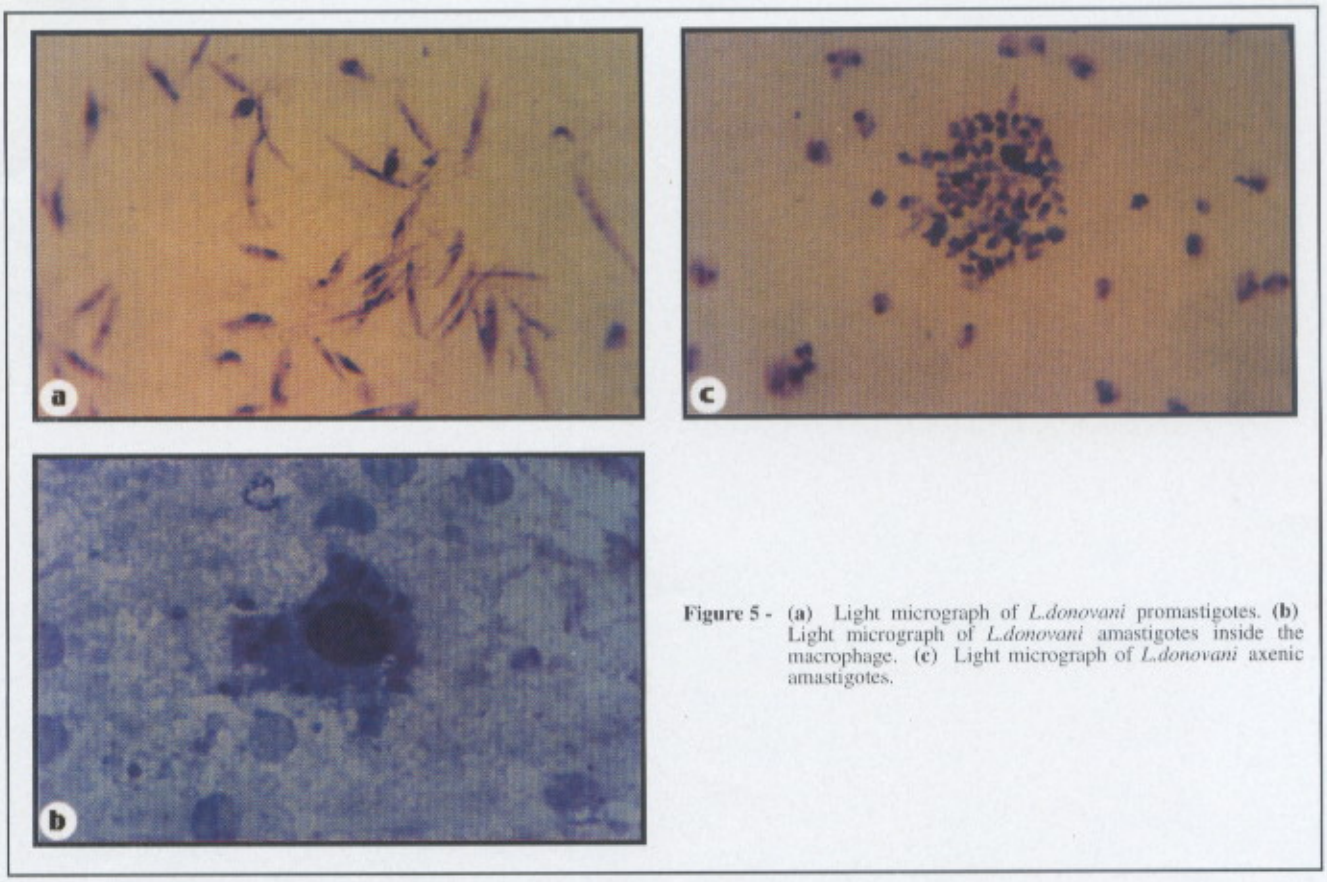

Figure 5 - **(a)** Light micrograph of *L.donovani* promastigotes. **(b)** Light micrograph of *L.donovani* amastigotes inside the macrophage. **(c)** Light micrograph of *L.donovani* axenic amastigotes.

round to oval cells, having approximately the same size and lacking an extension of the flagellum beyond the cell body (Figure 5a, 5b, and 5c). This agrees with the findings of others who investigated axenic cultivation of amastigotes of other species of *Leishmania*.[4,7] Several studies reported that promastigotes round up in shape when incubated at elevated temperature[18,19] but, it is important to distinguish between heat-shocked promastigotes and true amastigotes. Unfortunately, this cannot be accomplished by looking at morphology alone.

Ultrastructure. Another way used to distinguish amastigotes, axenic or tissue, from promastigotes is by studying their ultrastructure. This is a very useful tool, capable even of differentiating between some species of *Leishmania*.[20] A very useful marker to differentiate between amastigotes and promastigotes is the presence or absence of a paraxial rod. This crystalline structure has been described in several members of the family Trypanosomatidae.[21-24] We investigated the presence or absence of the paraxial rod in promastigotes and amastigotes under electron microscopy as described by Hayat.[25] Promastigotes have been shown to possess a paraxial rod running parallel to the flagellar axoneme. Vickerman and Tetley[23] reported that the paraxial rod is often not present until the flagellum has emerged from the flagellar pocket. In our study, it appeared that the paraxial rod can be found even in parts of the flagellum that are inside the flagellar pocket as well as in the part extending beyond the cell body. The paraxial rod was not seen in axenic or tissue amastigotes (Figure 6-11). This agrees with several reports on the ultrastructure of axenic amastigotes.[4,7,26] In addition, the flagellar pocket of axenic amastigotes was found to contain filamentous materials, which were not seen in promastigotes, which agrees with the work of Pimenta et al.[27]

Protein banding pattern. Biochemically, axenic amastigotes have been shown to be similar to tissue amastigotes and to differ from promastigotes in several aspects including proteinase activities,[7,28,29] nucleases[30] and several other enzymatic activities.[31] We observed the protein banding pattern of promastigotes, axenic amastigotes and tissue amastigotes after being analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by total protein staining with Coomassie Blue as described in Hames and Rickwood.[32]

It appeared that axenic amastigotes are similar to tissue amastigotes and different from promastigotes in protein banding pattern as revealed by SDS-PAGE. A variety of bands were observed in all forms including one prominent band. The identity of these proteins has not been determined. However,

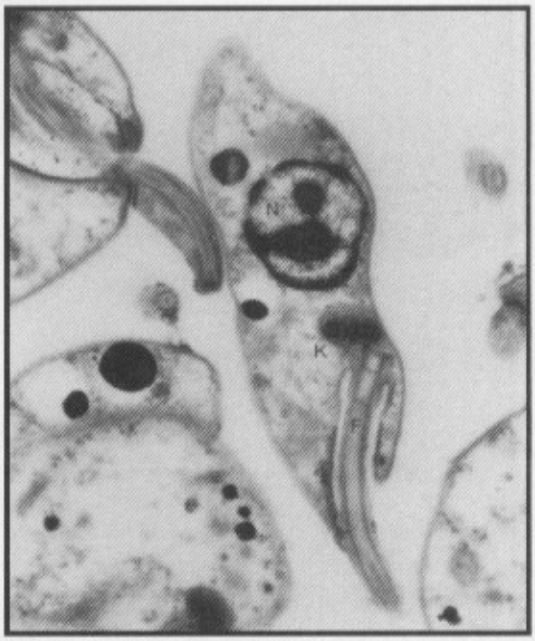

Figure 6 - Transmission electron micrograph of a longitudinal section of a *L.donovani* promastigote. N=nucleus, F=flagellum, K=kinetoplast. Bar = 260 nm.

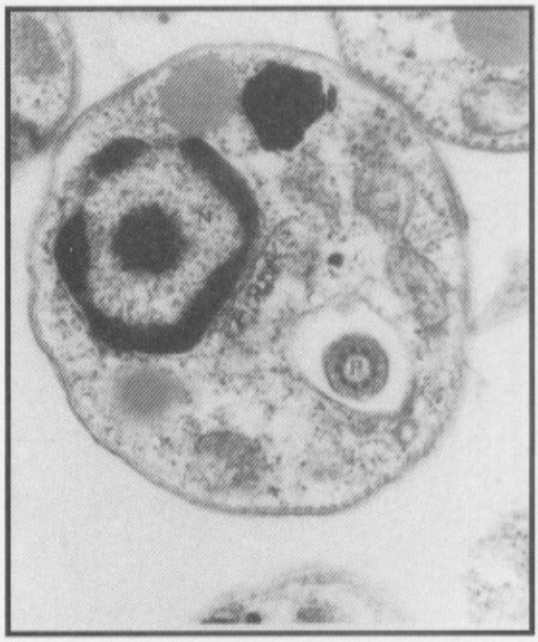

Figure 8 - Transmission electron micrograph of tissue amastigote showing the absence of the paraxial rod. N=nucleus, F=Flagellum. Bar = 140 mn.

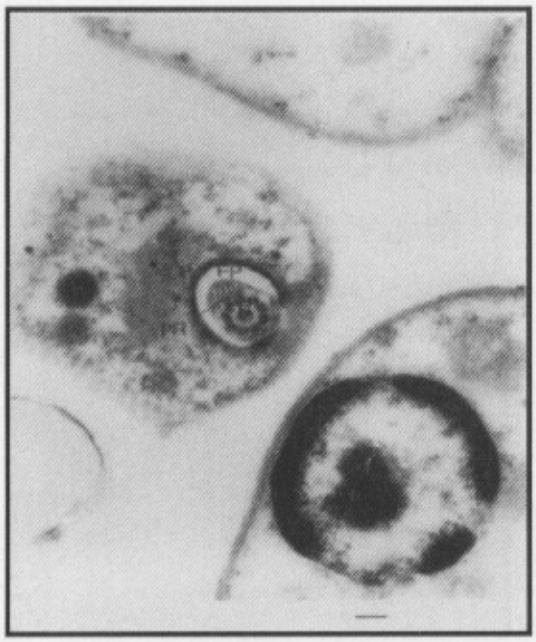

Figure 7 - Transmission electron micrograph of a cross section of a *L.donovani* promastigote showing the paraxial rod. F=flagellum, FP=flagellar pocket, PR=paraxial rod. Bar= 100 nm.

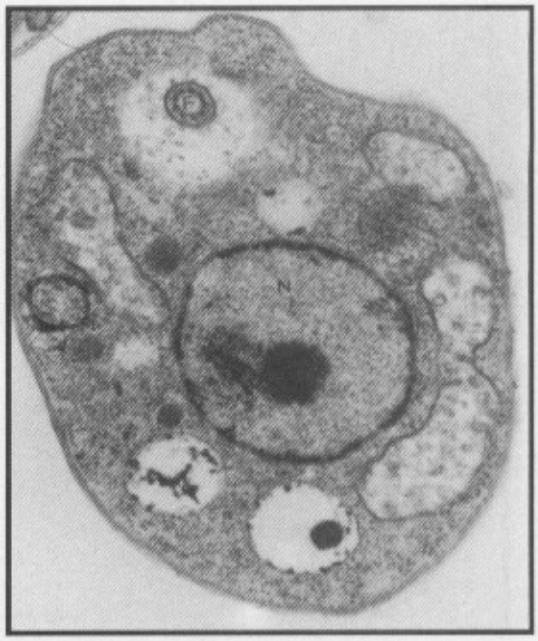

Figure 9 - Transmission electron micrograph of axenic amastigote showing the absence of the paraxial rod. N=nucleus, F=flagellum. Bar = 200 mn.

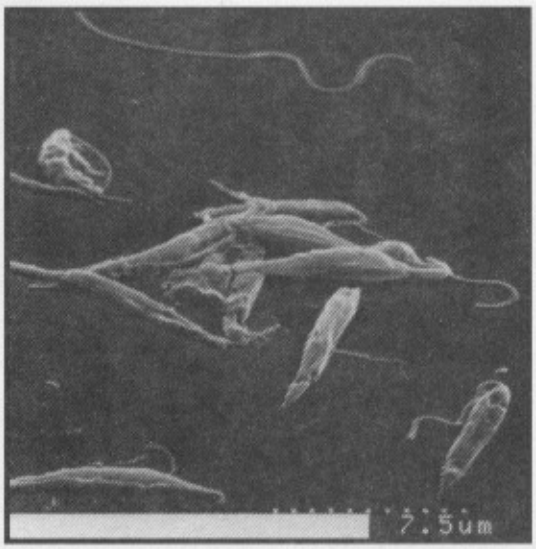

Figure 10 - Scanning electron micrograph of promastigotes.

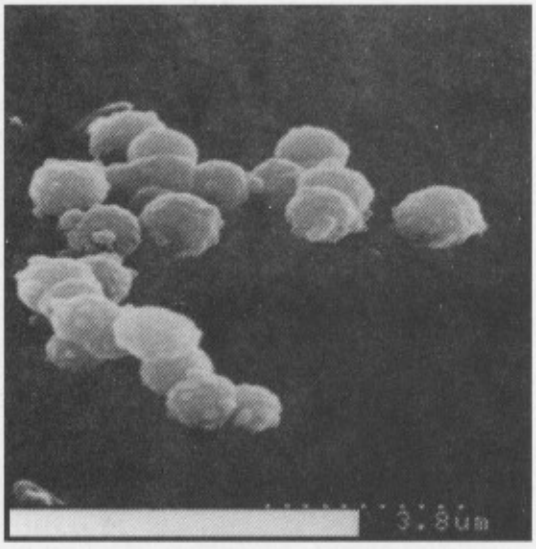

Figure 11 - Scanning electron micrograph of axenic amastigotes.

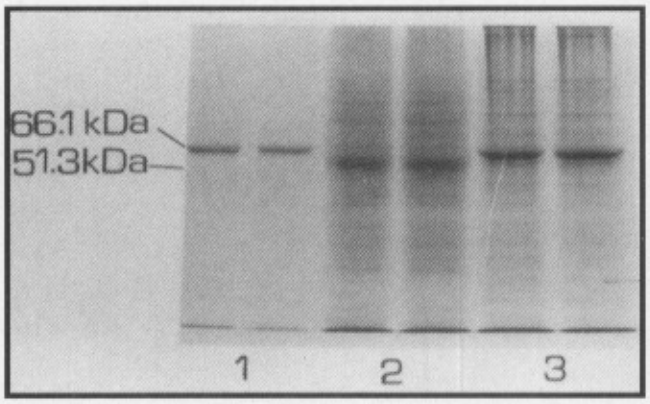

Figure 12 - Coomassie Blue staining of SDS-PAGE of lysates of axenic amastigotes (1), Promastogotes (2), and tissue amastigotes (3).

they are likely to be major structural proteins. The molecular mass of this band was approximately 51.3 kDa in promastigotes and 66.1 kDa in axenic amastigotes and tissue amastigotes (Fig. 12). The molecular weight of these bands is similar to that of ß-tubulin.[33] *L.mexicana* amastigotes have been shown to have a single-tubulin mRNA while promastigotes have ß-tubulin mRNA of 3 different sizes.[34]

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