Dose-dependent differential effect of hemin on protein synthesis and cell proliferation in *Leishmania donovani* promastigotes cultured *in vitro*

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Leishmania donovani requires an exogenous source of heme for growth and transformation. In *in vitro* culture of the free-living promastigotes, exogenously added hemin enhances cell proliferation. In this investigation, the question of the function of heme with particular reference to protein synthesis and cell proliferation has been addressed. The results of *in vitro* cell culture experiments demonstrated that hemin (10 μM) alone is suitable for supporting optimum level of protein synthesis, and thereby cell proliferation of promastigotes to an extent that it can replace fetal bovine serum. However, *in situ* labelling experiments along with Western blots revealed that high concentration of hemin (50 μM) reduced the level of protein synthesis in general and of *b*-tubulin in particular with a concomitant induction of hsp90, and induced consequent morphological changes that are observed during *in situ* transformation of promastigotes in mammalian macrophages. These results therefore suggest that sudden exposure to high concentration of heme in mammalian macrophages may be one of the key factors that trigger promastigote to amastigote transformation in *L. donovani*. Furthermore, hemin with its dual characteristic could be used as a tool to understand molecular mechanism of cell proliferation and transformation in these parasites.

1. Introduction

Leishmania donovani, which causes visceral leishmaniasis or kala-azar, exists in two developmental forms: a flagellated promastigote which lives in the gut of the sand fly vector, and a non-motile amastigote which lives in the phagolysosomes of mammalian macrophage. As is the case with other trypanosomatids, *Leishmania*, requires heme compounds as essential growth factor, due to lack of a complete heme biosynthesis pathway (Trager 1974). It has been demonstrated that *Leishmania* lacks two important enzymes required for heme biosynthesis, namely, aminolevulinate dehydratase and porphobilinogen deaminase. Therefore, exogenous source of heme in the form of hemin chloride, blood or serum is an obligatory requirement for growth of promastigotes and transformation of one form of the parasite to the other *in vitro* (Chang *et al* 1975; Chang and Chang 1985). This characteristic heme-dependency provide an insight into the underlying mechanisms controlling the differentiation of the parasites.

The precise role of heme in the proliferation as well as transformation of these parasites is unknown. Heme is utilized as a prosthetic group for many important proteins, such as, haemoglobin, catalase, and a number of proteins involved in the cytochrome system. More recently, heme in the form of hemin has been reported to act as an intracellular regulator of a variety of pathways for systems that utilize oxygen (reviewed in Padmanaban *et al* 1989). Further, requirement of heme for protein synthesis in mammalian reticulocytes and also in a number of non-erythroid cells is well established (see for reviews, Chen and London 1995; Pal *et al* 1996). Thus, heme is crucial for two very important physiological processes, namely, protein synthesis and respiration. Our aim has been to investigate the role of heme in the regulation of protein synthesis in *L. donovani*.

Here, the findings on the effect of heme on protein synthesis and proliferation of *L. donovani* promastigotes *in vitro* with particular reference to the changes in *b*-tubulin and hsp90 are reported. The results indicated that hemin at an optimum concentration of 10 μM supports normal protein synthesis and proliferation of promastigotes, while at a higher concentration (50 μM), it inhibits protein synthesis. At 50 μM hemin, the promastigotes

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tend to have a very short flagella and attain a rather stumpy from, perhaps an intermediate in the process of transformation to amastigotes. This process is accompanied by a down-regulation of *b*-tubulin and an up-regulation of an induced form of hsp90. These data therefore suggest that hemin could be utilized as a tool to understand development and differentiation of *L. donovani*.

2. Materials and methods

2.1 *Materials*

Cell culture media, RPMI 1640, and fetal bovine serum (FBS) were purchased from Gibco-BRL, USA. [^{35}S]methionine was obtained from BRIT (BARC), Mumbai. Hemin was from Sigma and all other chemicals used were from Sigma, Roche Molecular Biochemicals, Amersham and E-Merck. Promastigotes of *L. donovani* strains AG83 and UR6 (cloned lines) were obtained from the Cell Repository of National Centre for Cell Science, Pune. For all experiments reported herein except for *de novo* synthesis of proteins, strain AG83 was used. For the latter, strain UR6 was used.

2.2 *Cell culture and determination of optimum condition for cell proliferation*

L. donovani strains AG83 and UR6 were maintained in medium RPMI 1640 supplemented with 10% FBS, by subpassaging them every fifth day at 24°C. In order to determine the optimum concentration of hemin required for proliferation of promastigotes, $0{\cdot}5 \times 10^7$ promastigotes were inoculated in 5 ml of medium (without FBS) supplemented with various concentrations of hemin (0·5 μM–50 μM) and also without hemin supplement (as a control). Similar experiments were also carried out to determine the enhancing effect of hemin along with FBS on cell proliferation. Cells were counted at every 24 h for 4 days (in a haemocytometer), when the growth became stationary and the culture was ready for the next subpassage.

2.3 *Observation on morphology and behaviour of cells grown under various conditions*

For microscopic examination of Leishmanial morphology, the cells were stained in Giemsa as follows. Cells were smeared on glass slides, air-dried, fixed in methanol and stained in Giemsa for 5–8 min. Preparations were examined under light microscope and photographed. Live cultures as seen in phase contrast microscope were also photographed to check for the behaviour of cells grown under different conditions.

2.4 *Determination of effect of hemin on* de novo *protein synthesis by [^{35}S]methionine labelling*

The effect of hemin on *de novo* protein synthesis of the parasites grown in the presence of various concentrations of hemin and in FBS (control) was monitored by labelling the parasites for 1 h with 75 μCi of [^{35}S]methionine per 2 ml culture ($1{\cdot}5–2{\cdot}5 \times 10^7$ cells/ml). Water soluble proteins were extracted by sonication. Percentage incorporation of radioactivity in trichloroacetic acid (TCA) precipitable proteins was monitored on a scintillation counter. The protein samples with equal amount of radioactivity were analysed by SDS-PAGE (Laemmli 1970). The gel was processed for fluorography and exposed to X-ray film (Kodak X-Omat).

2.5 *Determination of effect of hemin on b-tubulin and hsp90 by Western blot analysis*

Proteins were extracted from the promastigotes grown under different conditions, estimated by Bradford's method (Bradford 1976), and were subjected to SDS-PAGE. The separated proteins in the gel were transferred on to nitrocellulose membrane according to Towbin *et al* (1979), and probed either with anti-rat *b*-tubulin or -HeLa hsp90 monoclonal antibodies. Protein signals were detected by using alkaline phosphatase-conjugated secondary antibody followed by colour detection or chemiluminescence detection as per the manufacturer's (Roche Molecular Biochemicals) protocol.

3. Results

3.1 *Hemin at a concentration of 10 mM supports optimum growth and proliferation of promastigotes, and can substitute FBS in* in vitro *culture*

In order to determine the optimum concentration of hemin required for promoting cell proliferation, various concentrations of hemin were used for cell culture without FBS supplement. Results obtained from four independent experiments indicated that hemin at an optimum concentration of 10 μM supports growth and proliferation of *L. donovani* promastigotes. Cells without any hemin supplement (control) did not grow and started dying within 24 h (data not shown).

Further, in order to determine to what extent hemin supports cell proliferation as compared to that by FBS, cell proliferation was determined by cell counts of cultures conditioned to three types of supplement, namely, 10 μM hemin, FBS, and FBS + 10 μM hemin, for a period of 3 days; cell counts were taken at every 24 h interval. As seen in figure 1, 10 μM hemin was able to support cell proliferation up to about 90% of what was done by FBS.

It was further evident that hemin added with FBS had a marginal enhancing effect (1·4%) on cell proliferation of *L. donovani* promastigotes as compared to that observed in the presence of FBS alone.

3.2 *Morphology and behaviour of cells grown under various conditions*

While culturing the promastigotes with various supplements as described below, some significant changes in morphology as well as behaviour were observed and the details are as follows. Figure 2 shows the Giemsa stained promastigotes and live culture observed under phase contrast microscope. Interestingly, in a number of experiments it was observed that the cells were more stout and longer when grown in the presence of 10 μM hemin alone (figure 2a) as compared to the cells grown in FBS (figure 2b) or FBS and hemin together (figure 2c). Furthermore, when grown in the presence of 10 μM hemin, the cells tended to cluster together through their flagella and float in the medium in the form of rosettes (figure 2d). This type of behaviour was also observed to some extent, among cells grown in the presence of both FBS and hemin. Such behaviour was infrequently observed in cells grown in FBS. At 50 μM hemin, however, the cultures could not be continued beyond a few days due to cell necrosis and therefore, the AG83 promastigotes could not be conditioned to 50 μM hemin. On the other hand, the promastigotes of the strain UR6 could be conditioned to 50 μM hemin, although their morphology changed (figure 3). The cells appeared less elongated (stumpy) and with short flagella (figure 3b) as compared to those grown in FBS (figure 3a).

3.3 *Effect of hemin on* de novo *synthesis of total soluble proteins*

Analysis of the total protein profiles of promastigotes grown in the presence of various supplements, namely, 10 μM hemin, FBS and FBS + 10 μM hemin, revealed no significant differences except minor quantitative variation of certain protein bands (results not shown). It was of interest therefore, to analyse hemin vs. *de novo* protein synthesis. The effect of hemin on *de novo* synthesis of proteins of the parasites (strain UR6) grown in the presence of various concentrations of hemin was monitored by labelling the parasites with [^{35}S]methionine. Parasites grown in medium supplemented with 10% FBS were used as control. Percentage of incorporation of radioactivity was less in 10 and 50 μM hemin supplemented cultures as compared to that in the control culture (table 1). In a subsequent experiment on detailed analysis, with various hemin concentrations, by SDS-PAGE followed by fluorography, it was evident that for this strain of parasites

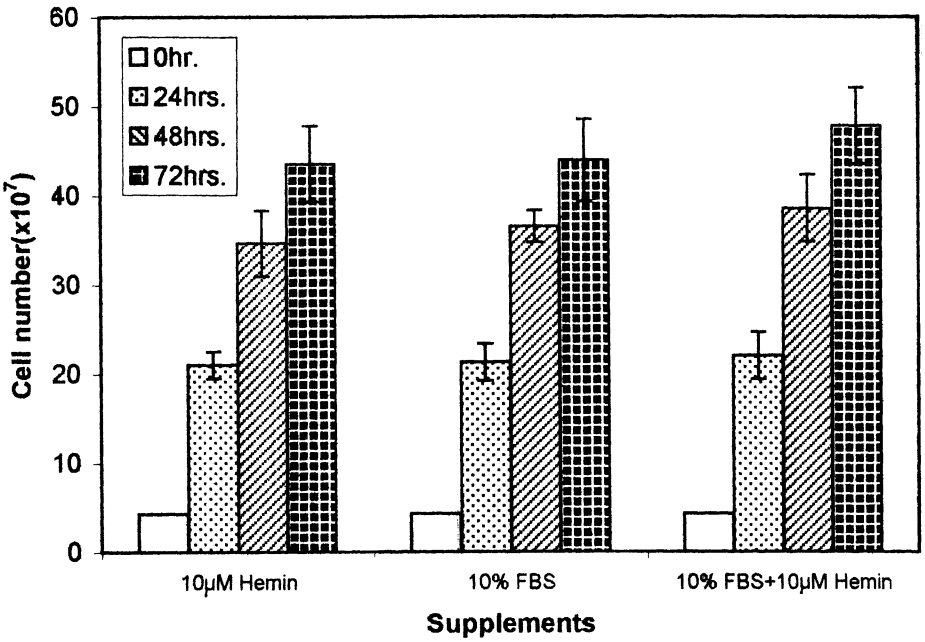

Figure 1. Hemin marginally enhances proliferation of promastigotes grown in the presence of 10% FBS. Promastigotes (4×10^7 cells) were inoculated in the medium RPMI 1640, and were grown for 3 days in the presence of various supplements, namely, 10 μM hemin, 10% FBS and 10% FBS + 10 μM hemin. Cell counts were taken at every 24 h for 3 days.

(UR6), the concentration of hemin required for optimum level of protein synthesis was less than 10 μM (figure 4). Furthermore, a drastic decrease in the quantity of putative *b*-tubulin subunit synthesis was observed in the parasites grown at 50 μM hemin (figure 4, lane 5). Although sample loading in each lane was normalized as per total cpm, at 50 μM hemin, in particular, less signal present in the form of bands may be due to protein aggregation as seen on the top of the lane. Further, the loading of 1 μM hemin-treated sample was slightly higher (figure 4, lane 3) as it appears in the autoradiogram profile. The overall results indicated that high concentration of hemin (50 μM), affected *de novo* protein synthesis significantly.

3.4 *Effect of hemin on quantitative variation of b-tubulin in the promastigotes*

As described above, 50 μM hemin affected putative *b*-tubulin biosynthesis. In order to further ascertain the nature of this band as *b*-tubulin, protein extracts from cells grown in FBS, 10 μM and 50 μM hemin and 10 μM hemin + FBS, were Western blotted with commercially available anti-rat *b*-tubulin antibody, which has been shown to recognize leishmanial *b*-tubulin (Werbovetz *et al* 1999). As seen in figure 5a, protein extracts of parasites grown in 10% FBS and 10 μM hemin supplement had *b*-tubulin signal; this signal appeared more intense in the parasites grown in 10% FBS + 10 μM hemin supplement. Further, another intense band of slightly lower molecular weight was also seen in the extracts of parasites grown in the presence of FBS + 10 μM hemin. It remains to be

Table 1. Percentage incorporation of [^{35}S] methionine in TCA precipitable proteins of promastigotes grown under various conditions.

Sample	CPM		Incorporation (%)
	Total	TCA	
Control (FBS)	49444	16489	33·34
Hemin 10 μM	45269	12847	28·37
Hemin 50 μM	11449	3053	26·66

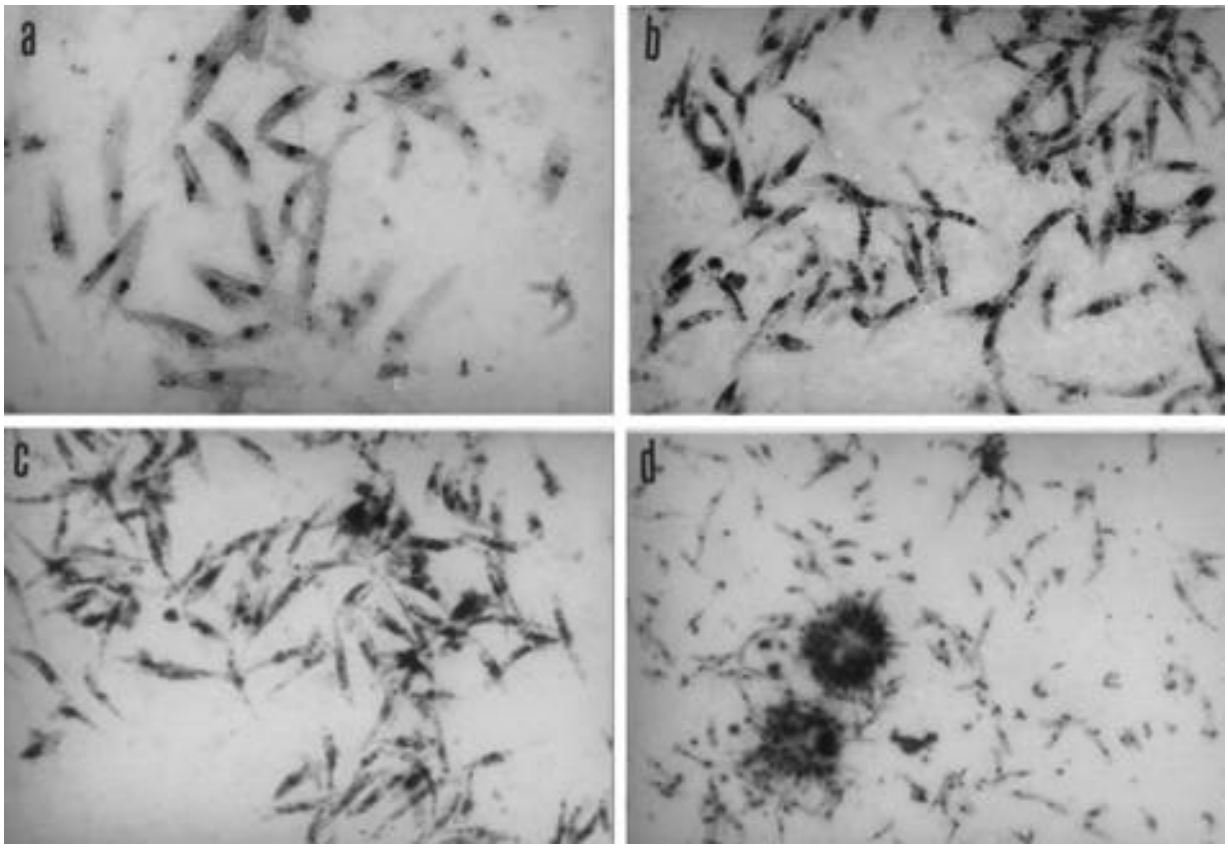

Figure 2. Morphology of *L. donovani* promastigotes grown under various conditions. Photographs of Giemsa stained smears (**a–c**) and live cultures (**d**) of promastigotes of *L. donovani* grown in RPMI 1640 with various supplements, namely, 10 μM hemin (**a, d**), 10% FBS (**b**) and 10% FBS + 10 μM hemin (**c**). Cells were observed under light microscope. Note the rosette-like structures which were observed only in cultures of promastigotes grown in the presence of 10 μM hemin. (**a–c**, × 330; **d**, × 132.)

established if this band represents another form of *b*-tubulin. On the other hand, in the extracts of the parasites grown in 50 μM hemin no *b*-tubulin signal was detectable. Thus, 50 μM hemin not only inhibits *b*-tubulin synthesis but it also induces preferential degradation of *b*-tubulin.

3.5 *Effect of hemin on the expression of hsp90*

In order to determine if high concentration hemin acts as a stress-inducing compound, the expression of hsp90 in the promastigotes grown in the presence of various supplements, as described above, was determined. Two intense bands in the form of a doublet could be detected in the cells grown in the presence of FBS (figure 5b, lane 3). In cells grown in the presence of FBS + 10 μM hemin, in addition to these two bands, a higher molecular weight form of hsp90 was also detected (lane 4). Interestingly, this higher molecular weight form was also seen in cells

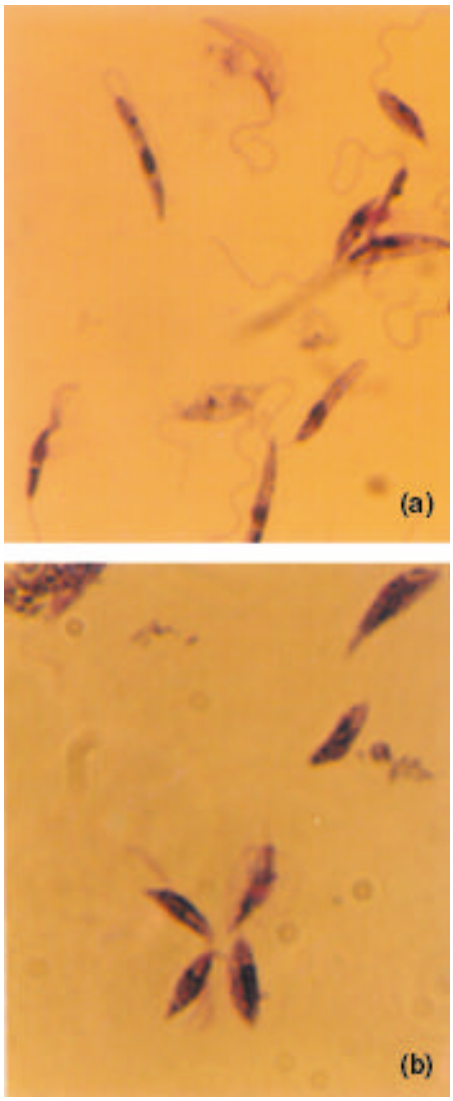

Figure 3. Morphology of promastigotes of *L. donovani* (strain UR6) grown in the presence of FBS and hemin. Giemsa stained smears of promastigotes grown for 4 days in 10% FBS (**a**) and 50 μM hemin (**b**). Cells were conditioned to FBS (**a**) and 50 μM hemin (**b**) for several generations. (× 330.)

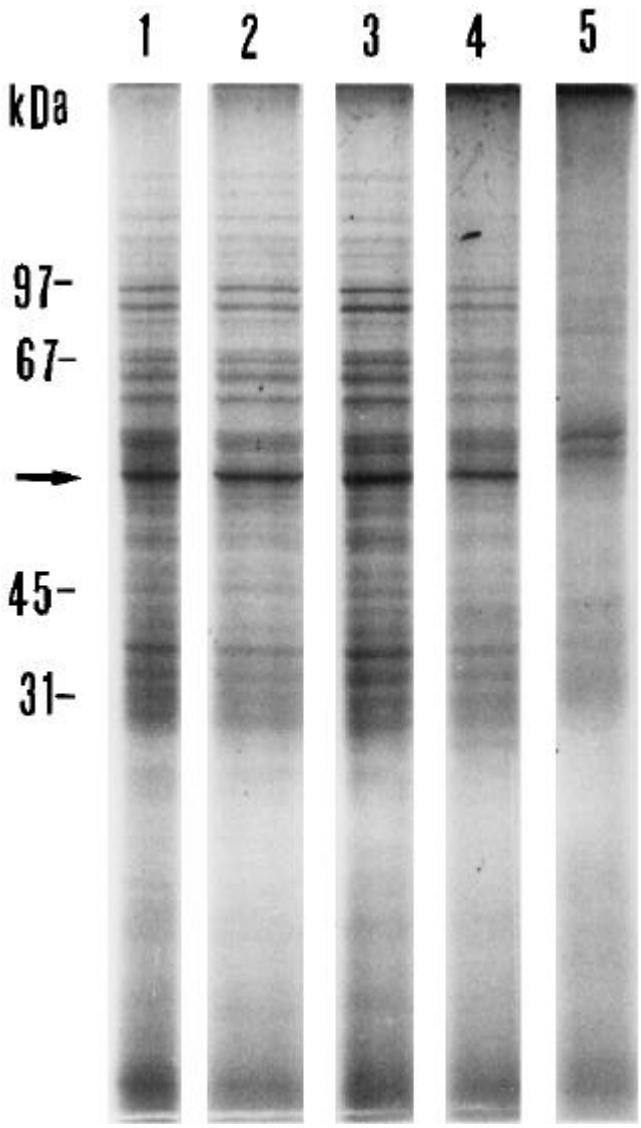

Figure 4. Effect of hemin on *de novo* synthesis of water soluble proteins of promastigotes of *L. donovani* (strain UR6), analysed by SDS-PAGE followed by fluorography. Samples containing protein extracts (2,80,000 cpm) of promastigotes grown in FBS (lane 1), and in hemin of various concentrations, namely, 0.1 μM (lane 2), 1.0 μM (lane 3), 10 μM (lane 4) and 50 μM (lane 5) were loaded in the gel. Positions of molecular weight marker proteins are indicated. Arrow indicates putative *b*-tubulin.

Figure 5. Effect of hemin on *b*-tubulin and hsp90 of promastigotes of *L. donovani* determined by Western blot using anti-*b*-tubulin (**a**) and anti-hsp90 (**b**) antibodies. Samples containing 100 µg of protein from promastigotes grown in the presence of various supplements, namely, 10 µM hemin (lane 1), 50 µM hemin (lane 2), 10% FBS (lane 3) and 10% FBS + 10 µM hemin (lane 4) were loaded in each lane.

grown in 10 µM hemin and 50 µM hemin, and in the latter, the intensity of this band was much higher.

4. Discussion

In this paper, the effect of hemin on protein synthesis and proliferation of *L. donovani* promastigotes cultured *in vitro* with a particular reference to the expression of *b*-tubulin has been reported. This is the first detailed study on the effect of hemin on protein synthesis and cell proliferation of *L. donovani* promastigotes. The results obtained in this investigation indicated that when *L. donovani* promastigotes are cultured *in vitro* without FBS, the optimum concentration of hemin required for growth of these parasites (strain AG83) is 10 µM, whereas the strain UR6 appeared to require lesser concentration. Ten µM hemin when added to the culture grown in the presence of 10% FBS was found to have an enhancing effect on the proliferation of *L. donovani* promastigotes. The rate of proliferation of the promastigotes grown in the presence of 10 µM hemin alone, was slightly lower as compared to that of parasites grown in FBS or FBS + hemin. There were only some minor differences in the protein profiles of these cultures. Therefore, these data indicate that 10 µM hemin could be used as a substitute for FBS in culturing these parasites.

At 50 µM hemin, a major protein, putatively *b*-tubulin, appeared to be selectively affected; no *de novo* synthesis was detected. In order to establish the nature of this protein as *b*-tubulin, protein extracts of the promastigotes grown in the presence of various supplements as described in the results were subjected to Western blot with anti-rat brain *b*-tubulin antibody. Results of this experiment seem to be intriguing. High concentration (50 µM) of hemin not only down-regulated *b*-tubulin synthesis but also led to its degradation. As seen in figure 5a, lack of *b*-tubulin signal at 50 µM hemin (lane 2) due to possible aggregation of *b*-tubulin was ruled out by the fact that the antibody did not detect any higher molecular weight protein band. Therefore, it is unclear how hemin leads to degradation of this protein. Although it is known that hemin acts as a protein stabilizer (Padmanaban *et al* 1989), its role as a destabilizer is unknown. Further experiments with purified *b*-tubulin *in vitro* may elucidate the mechanism.

With reference to the presence of another intensely stained signal of lower molecular weight with anti-*b*-tubulin antibody in the Western blot of cells grown in the presence of FBS + 10 µM hemin (figure 5a, lane 4), it is unclear whether it represents another form of *b*-tubulin. This band was only observed in the extracts of cells grown in the presence of FBS +10 µM hemin, when the promastigotes appeared healthy in morphology as well as cell proliferation. In *L. mexicana*, it has been reported that in the promastigotes, there are 3 different mRNAs for *b*-tubulin (Fong *et al* 1984). Similarly, in *L. major*, a set of 3 mRNAs for *b*-tubulin have been shown in promastigotes and these transcripts are expressed differentially during differentiation in correlation with acquisition of promastigote infectivity (Coulson *et al* 1996). It is therefore possible that in *L. donovani* promastigotes, this band may represent an isoform of *b*-tubulin translated from the corresponding mRNA of smaller size, under this specific condition of growth.

It was reported earlier that during transformation of promastigotes to amastigotes down-regulation of tubulin is preceded by up-regulation of heat shock proteins (Bard 1989), although the mechanism and the relationship between these two events has not been established yet. Therefore the level of hsp90 in promastigotes grown under various conditions including at 50 µM hemin was determined. As described in the results, in addition to two hsp90 bands, which are also seen in FBS grown cells, a slightly higher molecular weight protein band recognized by anti-HeLa hsp90 monoclonal antibody was detected both in 10 µM and 50 µM hemin grown cells, although the intensity of this band was significantly higher in 50 µM hemin. This band appears to represent an inducible isoform of hsp90. Such hemin-induced elevated expression of hsp90 has been shown in mammalian cells (Sistonen *et al* 1992). Thus, these two events of down-regulation of tubulin and up-regulation of hsp90 as observed in *L. donovani* promastigotes grown at high hemin concentration which is accompanied by morphological changes similar to what happens during transformation may indicate that high concentration of heme in macrophages *in vivo* could be one of the triggering factors for promastigote-amastigote transformation in the mammalian hosts.

In conclusion, these observations are of significant interest, not only in understanding the role of hemin in

cell proliferation and transformation, but also to explore the role of hemin as a tool to manipulate these parasites and control their growth and multiplication.

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