RESULTS
CHAPTER 3

Induction of stress proteins by the promastigote form of *L. donovani* virulent and avirulent strains upon heat shock and pH shock.
INTRODUCTION

Protozoan of the genus *Leishmania* are obligate intracellular parasites that cycle between the mid-gut of sandflies and the phagolysosomes of mammalian macrophages and therefore are exposed to extreme environmental changes (Zilberstein and Shapira, 1994). *Leishmania donovani*, the etiological agent of visceral leishmaniasis, commonly known as kala-azar is mainly endemic in the Eastern region of India. The parasite replicates in mononuclear phagocytic cells of the spleen, liver, lymph glands, bone marrow and produces a chronic disease. *L. donovani*, like other species of *Leishmania*, experiences a shift in temperature during its life cycle and differentiates from promastigote form in poikilothermic sandfly (at 25°C) to amastigote form in homeothermic mammalian host (at 37°C). The successful differentiation of the promastigote to the amastigote form is essential for the disease development. The most striking feature of all the leishmanial infection is their exclusive parasitization of the phagolysosomal compartment of the mammalian macrophages where the pH is between 4.5-6.0 (Luckacs *et al*, 1991). The survival of the *Leishmania* parasite during extreme variations in both the temperature and the pH is suggestive of an important role of heat shock proteins (HSPs)/stress proteins in *Leishmania* pathogenesis. The molecular events taking place in the promastigotes upon heat shock or encountering a low pH are poorly understood. Therefore understanding of the precise mechanisms that permit the survival and growth of the parasites under various cellular stresses such as heat shock and low pH are of great significance in order to develop protective strategies.
Cells were grown in Medium199 containing 10% FBS at 24°C. No. of parasites/ml were counted using a hemocytometer.
In this chapter, we have attempted to identify and characterize the stress proteins in promastigote form of *L. donovani*. Furthermore, the responses of the avirulent and virulent parasites were compared with respect to expression of HSPs.

RESULTS
Preliminary studies from our own laboratory indicated induction of certain HSPs in promastigotes of *L. donovani* following heat shock or pH shock. These studies were reconfirmed and extended further during the course of the present work. The responses of the virulent and avirulent parasites were also found to be significantly different.

Growth curve
A growth curve of *L. donovani* strain AG83 was prepared by suspending the promastigotes at the concentration of $1 \times 10^6$ cells/ml in medium M199 supplemented with 10% fetal calf serum (FCS) at 24°C (Fig. 2). Aliquots were withdrawn for counting after 24h intervals daily for up to 14 days and promastigotes number was determined using hemocytometer under phase contrast microscope. In common with other microorganisms cultured *in vitro*, a logarithmic growth phase was observed, followed by a period of slower growth, a stationary phase and eventually cell death leading to a decrease in density. Typically, no lag phase was observed, the log phase continued up to 7-8 days and the parasite entered the stationary phase thereafter. In order to maintain the *Leishmania* culture the promastigotes were subcultured every 3-4 days, typically in their log phase of growth. Since the late log phase or early stationary phase promastigotes are known to be more infective than log
phase promastigotes (da Silva and Sacks, 1987) therefore, 7-8 days old cultures were used during the experiments.

**Metabolic labelling:**
Axenically grown promastigotes of *L. donovani* from early stationary phase were suspended in methionine-deficient medium containing 20 % FCS (2.5-5 x 10^7 cells/ml). After equilibration at 24°C for 15 min, cells were metabolically labelled by incubating them with [³⁵S]-methionine for different time intervals namely 10 min, 60 min, 120 min and 180 min. The labelled parasites were washed thrice with cold medium and finally lysed in Laemmli's buffer. An aliquot was taken for determining the counts by scintillation counter. The time period of 2h was chosen for *in vivo* metabolic labelling for subsequent experiments since sufficient incorporation was obtained after 2h.

**Protein synthesis in virulent promastigotes on heat shock:**
Promastigotes of *L. donovani* were metabolically labelled as described above at 24°C and 37°C for 2h. Total cellular protein from an equal number of promastigotes was subjected to SDS-PAGE using 12 % gels according to Laemmli (1970). Standard molecular weight markers were run each time. Gels were stained with 0.1% Coomassie brilliant blue for 30min then destained using methanol acetic acid solution until clear visible bands were seen. To enhance the radioactive signal gels were further fluorographed using 20% PPO-DMSO solution, dried and then autoradiographed.
Figure 3: Analysis of protein profile of promastigotes of *L. donovani* upon heat shock. Promastigotes of virulent and avirulent strains were either maintained at 24°C, lane V2 and A2 respectively or subjected to heat shock at 37°C, lane V1 and lane A1, resolved by SDS-PAGE and stained with Coomassie. The number indicates the apparent molecular weight of the corresponding band determined according to mobility of standard molecular weight markers. Data shown is representative of one of the several independent experiments that yielded similar results.

Figure 4: Protein synthesis in virulent promastigotes on heat shock. Promastigote proteins were metabolically labelled with [³⁵S]-methionine at 24°C (lane A) or 37°C (lane B), resolved by SDS-PAGE and autoradiographed. The arrows indicate the position of the putative heat shock proteins and the numbers indicate the apparent molecular weight of the corresponding band determined according to mobility of standard molecular weight markers. The experiment was repeated four times with reproducible results.
The overall protein profile was not altered significantly upon heat shock as detected by Coomassie staining. *L. donovani* promastigotes grown at 24°C showed approximately 20 major and minor polypeptides in total, in molecular weight range of 100 to 14 kDa. The four major polypeptides with a Mr of 83, 70, 56 and 54 kDa were found to have a high constitutive level of expression (Fig. 3).

However, analysis of the radio-labelled promastigote proteins subjected to heat shock revealed a overall reduction in the total cellular protein synthesis. Heat shock led to an appreciable increase in the synthesis of certain proteins. Promastigotes subjected to 37°C for 2h showed induction of proteins of apparent Mr 110, 83, 70, 65, 40 and 12 kDa, whilst the synthesis of two major proteins of 56 and 54 kDa decreased considerably on heat shock (Fig. 4).

**Protein synthesis in avirulent promastigotes on heat shock:**
Metabolic labelling of avirulent promastigotes was performed as described previously. Promastigotes given a heat shock treatment at 37°C for 2h showed a greater decrease in total protein synthesis as compared to virulent strain (Fig. 5). Extracellularly grown promastigotes of avirulent strain also upon being subjected to heat shock showed elevated expression of putative HSPs 83, 70 and 65 (Fig. 5). However, we did not observe an increase in synthesis of proteins of 110, 40 and 12 kDa in the avirulent parasite upon heat shock.
Figure 5: Protein synthesis in avirulent promastigotes of *L. donovani* on heat shock. Promastigotes were subjected to heat shock at 24°C (lane 1) or 37°C (lane 2) proteins were labeled with $^{35}$S methionine. The labelled proteins were separated by SDS-PAGE and autoradiographed. The numbers indicate the position and apparent molecular weight of corresponding band determined according to the mobility of standard molecular weight markers. The experiment was repeated several times with reproducible results.

Figure 6: Protein synthesis in virulent promastigotes of *L. donovani* at different temperatures. Promastigotes were subjected to heat shock at 37°C (lane 1), 39°C (lane 2) or 41°C (lane 3), proteins labelled with $^{35}$S methionine were separated by SDS-PAGE and autoradiographed. The numbers indicate the the position and apparent molecular weight of corresponding band determined according to the mobility of standard molecular weight markers. The experiment was repeated several times with reproducible results.
Analysis of protein profile of virulent promastigotes at higher temperatures:

Heat shock treatment was carried out at three different temperatures 37°C, 39°C and 41°C. In addition to the normal physiological temperature of 37°C, the temperature of 39°C was selected, as *L. donovani* is known to prefer this slightly higher temperature for growth in a mammalian host where as a temperature of 41°C was chosen as it constitutes a heat shock for eucaryotic cells that grow normally at 37°C. Promastigotes treated at 39°C for 2h showed a greater increase in heat shock protein synthesis as compared to 37°C, control (Fig. 6). Heat shock at 39°C resulted in significant increase in expression of 110, 83, 70 and 65 kDa proteins (Fig. 6). However at 41°C, the relative protein synthesis by virulent promastigotes was remarkably lower as revealed by 35S methionine. Metabolic labelling of promastigotes at 41°C detected three visible bands of apparent Mr 83, 70, and 56-54 kDa proteins (Fig. 5).

Identification of heat shock proteins in response to low pH in virulent and avirulent promastigotes of *L. donovani*:

The pH value in the phagolysosomes varies in the range of pH 4.5 - 6.0, therefore it was decided to examine the effect of low pH (5.5) on stress protein synthesis by promastigotes of *L. donovani*. Since even the avirulent parasites were observed to be readily phagocytosed by macrophages in our *in vitro* model therefore, it was thought of interest to study the effect of low pH on the stress responses of the avirulent parasite as well. The protein synthesis in promastigotes of *L. donovani* in response to low pH was investigated by incubating promastigotes
**Figure 7:** Protein synthesis in virulent promastigotes of *L. donovani* in response to low pH. Promastigotes proteins were labelled with $^{35}$S methionine at 24°C (lane 1) or 37°C (lane 2) pH 7.4, (lane 3) pH 5.5. The labelled proteins were separated by SDS-PAGE and autoradiographed. The numbers indicate the position and apparent molecular weight of corresponding band determined according to the mobility of standard molecular weight markers. The experiment was repeated three times with reproducible results.

**Figure 8:** Analysis of protein profile of avirulent promastigotes on pH shock. Promastigote proteins were labelled using $^{35}$S methionine at either 24°C (lane 1), 37°C (lane 3) pH 7.4 or 24°C (lane 2) and 37°C (lane 4) pH 5.5, resolved by SDS-PAGE and autoradiographed. The numbers indicate the position and apparent molecular weight of the heat shock proteins. The experiment was repeated four times with reproducible results.
Figure 9: Immunological characterization of HSP70 of *L. donovani* using monoclonal antibody BRM-22. Promastigotes were either grown at 24°C (lane 1) or heat shocked at 37°C (lane 2) and processed for immunoblotting as described in materials and methods. The signal was visualized using ECL kit according to manufacture’s protocol.

Figure 10: Identification of HSP60 of *Leishmania donovani* by immunoblotting. Promastigotes of *L. donovani* were propagated at 24°C (lane 1) or given heat shock treatment at 37°C (lane 2) and processed for immunoblotting as described in materials and methods section. The blot was probed using antibody to HSP60 (SPA-805) and the signal was visualized using ECL kit. Data shown for immunoblot analysis are representative of three independent experiments that yielded identical results.
(2.5-5 x 10^7 cells/ml) in presence of ^35_S-methionine for 2h at desired temperature. Virulent promastigotes when exposed to low pH 5.5 at 37°C showed a marginal increase in relative synthesis of 110, 83 and 70 kDa proteins as compared to control (Fig. 8). Avirulent promastigotes subjected to low pH 5.5 showed a decrease in total protein synthesis both at 24°C as well as 37°C with a relative increase in synthesis of HSP70 and 83 only (Fig. 8). The induction of HSPs by avirulent promastigotes was more marked at 37°C as compared to 24°C.

**Immunological characterization of HSP70 and HSP60:**

Immunological characterization of HSP70 and HSP60 was carried out by Western blotting. Protein extracts from control as well as heat shocked promastigotes gave a band corresponding to Mr of 70-kDa when tested with monoclonal antibody against a mammalian HSP70. The intensity of signal was higher in heat shocked cells as compared to control (Fig. 9). Immunoblotting performed with HSP60 (SPA-805) showed a single band corresponding to molecular size of 65 kDa in control as well as heat shocked preparations. The signal generated in response to heat shock was only marginally higher than the control cells (Fig. 10).

**DISCUSSION**

HSPs serve vital biochemical functions in the cell even in the absence of stress. They play a major role in folding, unfolding and translocation of proteins in the cell. Throughout its life cycle, *Leishmania* parasites encounter stressful conditions and have evolved various strategies against stresses in order to survive. The adaptation of *Leishmania*
parasite for survival over a wide temperature range suggests that heat shock responses may play a vital role in parasite differentiation/survival. Exposure of promastigotes to elevated temperatures in vitro has indeed been shown to result in the increased expression of classical heat shock proteins (HSPs) in a number of Leishmania spp (Hunter et al, 1984; Lawrence & Robert Gero, 1985).

A high constitutive expression of HSP70 and 83 kDa proteins has been observed in axenically grown promastigotes at 24°C in both virulent and avirulent strains of L. donovani. A two fold increase in synthesis of major HSPs i.e 70 and 83 was noted upon heat shock. In keeping with their general role as molecular chaperone, HSPs in Leishmania are probably involved in renaturation of stress-damaged proteins and are therefore required throughout the parasite life-cycle.

The virulent promastigotes of L. donovani were found to preferentially synthesize the putative HSPs of apparent Mr 110, 83, 70, 65, 40 and 12 kDa upon heat shock at 37°C. On the other hand, the avirulent parasite showed induction of HSPs 83, 70 and 65 only. The increased expression of certain HSPs in response to heat shock which the avirulent promastigotes failed to overexpress is suggestive of a role of stress proteins as virulence factor in Leishmania. Prior work has also detected an increase in virulence in promastigotes of Leishmania braziliensis following brief heat shock treatment (Smejkal et al, 1988), the molecular events induced in promastigotes which accounted for their increased virulence following heat shock were not very well delineated,
however overexpression of HSPs are likely to be involved in this phenomenon.

Temperature increase has also been shown to influence the stage differentiation of *Leishmania* and in certain species is sufficient to induce morphological alterations observed during transformation from promastigote to the amastigote form in axenic conditions (Van der Ploeg *et al*, 1985). Of particular interest, is the identification of small HSPs in *Leishmania* since these low molecular mass HSPs have been implicated to have a role in the differentiation process. The preferential synthesis of HSPs 40 and 12 kDa only by virulent promastigotes of *L. donovani* indicate a role of these HSPs in the differentiation process. HSP83 along with other small HSPs have been demonstrated to have a role in the differentiation of *Drosophila* (Bond and Schlesinger, 1987).

The transformation of parasite in mammalian macrophages involves a series of steps poorly understood at present. An initiating step in this process may be induction of HSPs by the parasite in response to increased temperature in the mammalian host. Macrophages may engulf the parasite already in the process of differentiation (Smejkal *et al*, 1988). HSPs induced in the promastigotes by nutritional deprivation in the insect vector have been suggested to pre-adapt the parasite for stress on encountering the mammalian host (Toye and Remold, 1989). Likewise, the HSPs induced upon heat shock may pre-adapt the promastigotes for stresses it encounters inside the macrophages. The increased expression of low molecular mass HSPs (40 and 12 kDa) besides 110 kDa protein only in the virulent strain suggests that these
proteins in particular may have a role in pre-adapting the *Leishmania* parasite for survival/differentiation inside the host macrophages.

An increase in body temperature is a characteristic symptom of visceral leishmaniasis. A greater induction of HSPs in virulent promastigotes of *L. donovani* at 39°C point towards an important role of HSPs in *L. donovani* survival in the visceral organs of their mammalian host.

Another major stress that *Leishmania* encounter in the macrophage is the high proton concentration within the phagolysosomal compartment where the pH is between 4.5 to 6.0. The precise mechanism(s) that permit the survival and growth of *Leishmania* parasite in acid environment are of great significance. However, the molecular details of the events taking place in promastigotes on encountering such a low pH are not very well understood. Although it has been demonstrated that most of the metabolic activities of promastigotes decrease at pH below 5.0 (Zarley *et al*, 1991). Phagosome acidification to pH below 5.0 has been implicated as an intracellular inducer of activation of virulence gene expression in *Salmonella* and the enhanced expression of selected proteins has been shown to contribute to bacterial survival within macrophages (Buchmeier and Heffron, 1990).

Exposure of the promastigotes of *L. donovani* to low pH 5.5 in combination with heat stress resulted in differential expression of HSPs by two strains of *Leishmania* parasite. Unlike, the virulent
promastigotes the avirulent parasite did not show any detectable increase in HSP110 and 65. In an earlier study the promastigotes of *L. donovani* upon being subjected to a low pH 4.5 in conjunction with heat shock showed an increase in relative synthesis of HSP110, 83, 70 and 65 while the avirulent parasite exhibited a remarkable increase in expression of HSP70 only (Salotra *et al.*, 1994). Therefore it appears that HSP110, along with other HSPs particularly HSP83 may be playing an essential role in virulence/survival/differentiation of *L. donovani* inside the phagolysosomes of infected host.

Immunological analysis of HSP 70 and 65 detected a marginal increase in expression of these proteins upon heat shock, although a two fold increase in synthesis of HSP70 was observed during $^{35}$S labelling experiments. The discrepancy in the results could be due to the use of two different techniques. $^{35}$S labelling reveals the profile of the proteins being actively synthesized during labelling period where as Western blotting shows a picture of total protein accumulated over a period of time. Since unusually high intracellular pool of HSP70 exist even in the unstressed promastigotes representing almost 2.1% of the total cell protein, increase in relative synthesis of HSP70 detected using $^{35}$S may not be sufficient to significantly affect total protein content upon heat shock.