CHAPTER 4

Evaluation of stress response of *L. donovani* promastigotes in response to cytokines and other effector molecules.
INTRODUCTION:

Intracellular replication of *L. donovani* inside macrophages is essential for production of disease and development of parasite. Cell mediated immunity is believed to play a key role in the host resistance against *Leishmania* infection. The outcome of the infection is closely linked to the pattern of cytokines produced by *Leishmania* reactive CD4+ T helper cells (Fig. 11) (Coffman *et al*., 1991). Susceptibility to infection is associated with activation of Th-2 cells secreting IL-4, IL-5, IL-6 and IL-10. In contrast, when T cell response is predominated by Th-1 cells producing IL-2, IFNγ, and lymphotoxin, the animal succeeds in eliminating the parasite and controlling the infection.

The role of cytokines in regulating *L. donovani* infection in humans is supported by the finding that elevated levels of IL-4 occur in plasma of kala-azar patients and by promising results of IFNγ inclusion in therapy of the disease (Badaro *et al*., 1990; Zwingenberger *et al*., 1990). Nitric oxide (NO) has been demonstrated to be the principal effector molecule in the killing of *Leishmania* by macrophages. The enhanced killing of intracellular *Leishmania* mediated by IFNγ and TNF-α involves increased synthesis of NO (Green *et al*., 1990, 1994). In contrast, other cytokines such as IL-4, IL-10 and TGF-β down-regulate the production of NO (Barral *et al*., 1993).

It is evident from recent studies that cytokines i.e. IFNγ and TNF-α can have a direct cytostatic effect on the growth of promastigotes of *L. donovani* (Bhattacharya *et al*., 1993). Since promastigotes of *Leishmania* are exposed to various cytokines
Figure 11: Development of the host immune response to *Leishmania* infection. Effective Th1 cell response on the left, ineffective Th2 cell response on the right. (Adapted from Locksley RM and Scott P, 1991 Immunology Today 12:A58-A61)
Induction

Peptide 'X' + IFN-γ + ???

T_H0

Peptide 'Y' + IL-4 + ???

T_H2

Expansion

T_H1

T_H1

T_H2

Effect

IL-2

TNF

IFN-γ

Macrophage activation resolution of disease

IL-4

IL-10

No macrophage activation progressive disease

Macrophage activation resolution of disease

Macrophage activation resolution of disease
subsequent to their inoculation into mammalian host, therefore, it becomes meaningful to study the effect of cytokines on the stress proteins synthesis by promastigotes. Cytokines viz. γIFN, TNF-α, IL-4, TGF-β were chosen in order to determine their effect on protein synthesis of virulent and avirulent parasite strains. Furthermore, we have examined the effect of antimicrobial factors i.e. H$_2$O$_2$, NO on stress proteins induction by the promastigote form of *L. donovani* virulent and avirulent strains.

**RESULTS:**

Preliminary studies from our own laboratory on the role of cytokines on stress-protein induction have demonstrated that treatment with Tumor necrosis factor alpha (TNF-α) led to an over-expression of HSPs by promastigotes of *L. donovani* virulent strain only. Results of previous studies were re-confirmed during the course of present work and further work in this direction was carried to find out the effect of various cytokines and antimicrobial factors i.e. Interferon gamma (IFNγ), Transforming growth factor beta (TGF-β), Interleukin four (IL-4), Hydrogen peroxide (H$_2$O$_2$) and Nitric oxide (NO) on the protein synthesis of virulent and avirulent promastigotes of *L. donovani*. 
Susceptibility of virulent and avirulent promastigotes to TNF-mediated toxicity:
Stationary phase promastigotes (metacyclic form) of *L. donovani* were suspended in growth medium M199 containing 20% FCS at the concentration of $10 \times 10^6$ cells/ml. To study the effect of TNF-α on the growth of promastigotes, cells were incubated with different concentrations of TNF-α for 2h at 37°C. Mobility of promastigotes is known to correlate with their survival. The viability of parasites was examined microscopically according to flagellar motility by counting a minimum of 200 promastigotes as motile (viable) or non-motile (non-viable). The survival of the virulent as well as avirulent promastigotes remained unaffected by the presence of TNF-α up to 1000 U/ml under the experimental conditions employed. At the end of 2h exposure to TNF more than 95% promastigotes remained motile.

Protein synthesis in virulent and avirulent promastigotes of *L. donovani* in response to TNF:
Promastigotes of *L. donovani* were metabolically labelled by incubating them with 200μCi/ml of $[^{35}S]$-methionine at 37°C for 2h in presence of various concentrations of TNF-α. After the incubation was over, cells were lysed in 2x SDS lysis buffer. The total cellular protein from an equal number of cells was resolved by 12% SDS-PAGE according to Laemmli (1970). Standard molecular weight markers were run each time. Gels were then fluorographed, dried and autoradiographed. A remarkable decrease in protein synthesis (as measured by incorporation of $^{35}$S methionine) was observed in the presence of TNF-α in a dose-
Figure 12: Protein synthesis in virulent and avirulent promastigotes in the presence of TNF. 1x 10^7 cells were metabolically labelled with ^35_S methionine at 37°C in the presence of 0, 250 or 500U/ml of TNF (lanes A,B,C respectively) for virulent and (lanes D,E,F respectively) for avirulent promastigotes. Total cellular protein from an equal number of parasite was resolved by SDS-PAGE and fluorographed. The arrows indicate the position of the putative HSP 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 three times with reproducible results.
dependent manner. The decrease in protein synthesis was more marked in avirulent than in the virulent promastigotes (Fig. 12). In the presence of 500 U/ml of TNF-α the virulent parasites showed an appreciable decrease in cellular protein synthesis with the preferential synthesis of HSP 83, 70 and 65. At 500 U/ml of TNF-α, the avirulent promastigotes showed negligible protein synthesis except for barely detectable level of HSP70 (Fig. 12). However, at 250 U/ml of TNF-α, a relative increase in synthesis of HSP83 and HSP70 over most cellular proteins was noted in avirulent parasite, the expression of HSP83 being significantly lower than HSP70.

Role of NO during Leishmaniasis:

NO is an essential mediator for a variety of biological functions, including defense against a range of pathogens. Induction of NO to cytotoxic levels by IFN-γ and TNF-α has been widely accepted as the principal effector mechanism of murine macrophages for destruction of Leishmania parasite (Liew 1990; Green et al, 1990). Furthermore, NO generated in response to heat shock has actually been shown to activate the stress protein synthesis in several mammalian organs (Malyshev et al, 1995). Our interest was to assess the effect of NO on synthesis of heat shock proteins by promastigotes of L. donovani. Promastigotes of L. donovani show optimal growth at 24°C and upon entry into mammalian host they are exposed to multiple cellular stresses viz. high temperature 37°C (physiological temperature of mammalian host) and antimicrobial factors including NO. Keeping this in mind, we attempted to study the effect of NO on HSPs expression of promastigotes at 24°C.
and 37°C. The responses of the virulent and avirulent strains exposed to NO were also compared.

**Susceptibility of promastigotes of *L. donovani* to NO mediated cytotoxicity:**
To test the effect of NO on promastigotes of *L. donovani*, sodium nitrite (NaNO₂) was utilized as a substrate. At an acidic pH, NO₂⁻ is converted to nitrous acid, subsequent decomposition of nitrous acid generates reactive nitrogen intermediates (RNIs), mainly NO (O'Brien et al., 1994). Since NO is considered to be the most potent RNI, we tested the effect of acidic as well as physiological NO₂⁻ solutions on the growth and HSPs expression of promastigotes. Cells (10-20 x 10⁶ cells/ml) were incubated in the presence of various concentrations of NaNO₂ ranging from (0 to 350 µg/ml) at 37°C for 3h. Both, the virulent and avirulent strains were observed to retain their viability (as indicated by flagellar movement under the microscope) in the presence of NaNO₂ (pH 5.0) up to 150 µg/ml concentration under the employed experimental conditions. A dose of 200 or 350 µg/ml (pH 5.0) resulted in almost 100% killing of promastigotes. However, promastigotes of *L. donovani* were observed to be killed even at pH 7.4, when exposed to NaNO₂ at the concentration of 1 mg/ml.

**Effect of NO on stress protein synthesis of virulent promastigotes.**
Stationary phase promastigotes of virulent and avirulent strains were metabolically labelled at 37°C using [³⁵S] methionine in presence of sublethal doses of NaNO₂ (125,150 µg/ml) at pH for 3h. Cells were lysed in SDS lysis buffer and total cellular protein from an equal
Figure 13: Analysis of protein profile of virulent promastigotes in presence of 'NO' at 37°C. Promastigotes of *L. donovani* were labelled with $^{35}$S methionine at pH7.4 in the presence of 0, 125 and 150 µg/ml of NaN0₂ (lanes 1, 2 and 3 respectively) or at pH5.0 (lanes 4, 5 and 6 respectively), resolved by SDS-PAGE and autoradiographed. The numbers indicate the position and 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.

Figure 14: Protein synthesis by avirulent promastigotes of *L. donovani* in response to NO at 37°C. Promastigotes were labelled with $^{35}$S methionine at pH 7.4 in the presence of 0 and 150 µg/ml of NaNO₂ (lanes 1, 2) or in the presence of 0, 150 and 125 µg/ml of NaNO₂ at pH 5.0 (lanes 3, 4 and 5 respectively), resolved by SDS-PAGE and autoradiographed. The numbers indicate the position and the apparent molecular weight of the corresponding band determined according to mobility of standard molecular weight markers. The experiment was repeated thrice times with reproducible results.
number of cells was resolved by 12% SDS-PAGE. Standard molecular weight markers were run each time. Gels were fluorographed, dried and autoradiographed. Promastigotes of *L. donovani* grown at 37°C, pH 5.0 showed considerable decrease in total cellular protein synthesis upon NO treatment. A substantial reduction was also noted in expression of two major promastigote proteins of 56 and 54 kDa (earlier identified as α and β tubulin respectively) in presence of NO (Fig. 13). However, we did not observe any appreciable change in protein synthesis by virulent promastigotes in presence of NaNO₂ solution at pH 7.4 (Fig. 13).

A marked increase in the relative synthesis of HSPs was shown by promastigotes exposed to NO. NO generated from NaNO₂ at pH 5.0 led to an overexpression of HSPs 65, 70 and 83 in virulent parasite in a dose dependent manner (Fig. 13). The relative synthesis of HSP70 and 83 was of greater magnitude than HSP65 (Fig. 13).

**Stress protein synthesis by avirulent promastigotes of *L. donovani* exposed to NO:**
A remarkable decrease in synthesis of total cellular protein was observed in avirulent promastigotes in presence of NO at 37°C, pH 5.0. The reduction was more marked in avirulent than the virulent strain indicating that the avirulent promastigotes were more susceptible to NO treatment (Fig. 14). Avirulent promastigotes exposed to NO showed a preferential expression of HSP83 and 70 over most other cellular proteins in a dose dependent manner. However, differential induction of HSPs by avirulent promastigotes during NO treatment was observed, synthesis of HSP70 being much more prominent than HSP83 (Fig. 14).
Figure 15: Protein synthesis in virulent promastigotes of *L. donovani* in response to NO at 24°C. Promastigotes were labelled with $^{35}$S methionine at either pH 7.4 in the presence of 0, 125 and 150 µg/ml of NaNO$_2$ (lanes 1, 2 and 3 respectively) or in the presence of 0, 125 and 150 µg/ml of NaNO$_2$ at pH 5.0 (lanes 4, 5 and 6 respectively). Promastigote proteins from control samples (lane 1-4) were diluted 1:2 times, resolved by SDS-PAGE and autoradiographed. The numbers indicate the position and the apparent molecular weight of the corresponding band determined according to mobility of standard molecular weight markers. The experiment was repeated three times with reproducible results.

Figure 16: Protein synthesis by avirulent promastigotes of *L. donovani* in response to NO at 24°C. Promastigotes were labelled with $^{35}$S methionine at pH 7.4 in the presence of 0 and 125 µg/ml of NaNO$_2$ (lanes 1, 2) or in the presence of 0, 125 and 150 µg/ml of NaNO$_2$ at pH 5.0 (lanes 3, 4 and 5 respectively), resolved by SDS-PAGE and autoradiographed. The numbers indicate the position and the apparent molecular weight of the corresponding band determined according to mobility of standard molecular weight markers. The experiment was repeated two times with reproducible results.
NaNO₂ solutions at pH 7.4, did not significantly alter the protein synthesis of HSPs by avirulent promastigotes of L. donovani (Fig. 14).

**Effect of NO on HSPs expression of virulent and avirulent promastigotes at 24°C:**

Treatment of virulent promastigotes at 24°C in presence of NaNO₂ at pH 5.0 revealed appreciable decrease in total protein synthesis. But, significant increase in relative synthesis of heat shock proteins (HSP70 and 83) occurred in presence of NO in a dose dependent manner (Fig. 15). Avirulent promastigotes treated under similar conditions showed a considerable decrease in total cellular protein synthesis (Fig. 16). A marginal increase in expression of HSP70 along with a barely detectable level of HSP83 was observed when exposed to a lower dose of (125μg/ml of NaNO₂) of NO. However, the synthesis of HSPs was negligible when exposed to 150μg/ml of NaNO₂ at pH 5.0.

**Stress proteins induction by virulent and avirulent promastigotes of L. donovani in response to γIFN:**

To test the effect of γ-IFN on protein synthesis of L. donovani promastigotes, stationary phase (infective form) promastigotes were labelled with ³⁵S-methionine in presence of various concentrations of γIFN (50 U/ml to 1000 U/ml) at 37°C for 2h. After the incubation period was over, cells were washed thrice in complete medium (containing methionine) and lysed with 2x SDS lysis buffer. Total cellular protein from an equal number of parasites was fractionated by SDS-PAGE. The gels were fluorographed, dried and autoradiographed.
**Figure 17:** Protein synthesis in virulent promastigotes during γIFN treatment. Promastigotes of *L. donovani* were maintained at 24°C (lane 1) or 37°C (lane 2) or treated with 50, 250, 500 and 1000U/ml of γIFN either by preincubation at 24°C for 24h and then subjected to heat shock (lanes 3, 4, 5, 6) or directly (at 37°C for 2h) (lanes 7, 8, 9, 10 respectively) in presence of $^{35}$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 three times with reproducible results.

**Figure 18:** Protein synthesis by avirulent promastigotes of *L. donovani* in presence of γIFN. 2x10^7 promastigotes were metabolically labelled with $^{35}$S methionine either at 24°C (lane 1) or heat shocked at 37°C in the presence of 0, 50, 250, 500 and 1000 U/ml of γIFN (lanes 2, 3, 4, 5, 6 respectively). Labelled protein from an equal number of parasites was resolved by SDS-PAGE and fluorographed. The arrows indicate the position of the HSPs 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 two times with reproducible results.
The image shows a gel electrophoresis pattern with molecular weight markers at 83 kDa and 70 kDa. The gel is labeled with kDa and numbered from 1 to 10. Each lane contains a sample, with variations in band intensity and position. The gel is used to analyze the molecular weight and expression of proteins.
Presence of γIFN did not result in any appreciable change in the expression of proteins. However, the control samples represented by extracellularly grown promastigotes at 37°C, revealed an enhanced synthesis of HSPs of 70, 83 and 65. The relative increase in HSP70 and 83 was much greater than 65 kDa (Fig.17). The above experiment was repeated several times with consistent results. It was thought that 2h time period may not be sufficient for the γIFN to act on the promastigotes, although this time period has been observed to be enough for the induction of stress proteins at 37°C. Therefore, promastigotes were preincubated with γIFN at 24°C for 24h and then subjected to heat shock at 37°C for 2h in presence of γIFN (Fig17). None of the doses of γIFN tested, effected the synthesis of HSPs by virulent or avirulent strain (Fig. 17 and 18).

**Evaluation of stress response in presence of TGF-β:**

To examine the effect of TGF-β on stress protein synthesis, stationary phase promastigotes of *L. donovani* virulent and avirulent strains were incubated with TGF-β at the concentration of (0.1 to 5ng/ml) at desirable temperature for 2h. The protein synthesis in presence of TGF-β was followed using ^35^S-methionine. Cellular protein from an equal number of promastigotes was resolved by SDS-PAGE. Gels were stained, destained, flurographed, dried and then autoradiographed as described previously. Promastigotes of *L. donovani* virulent and avirulent strains were found to be non-responsive to TGF-β treatment (Fig. 19). None of the doses of TGF-β tried, resulted in a significant induction of HSPs (Fig. 19).
Figure 19: Protein synthesis in virulent promastigotes in response to TGF-β. Promastigote proteins were labelled with \( ^{35} \text{S} \) methionine at 37°C in presence of 0, 0.1, 0.5, 2 and 5ng/ml of TGF-β (lanes 1,2,3,4,5 respectively). The labelled proteins were separated by SDS-PAGE and autoradiographed. The arrow and 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 20: Analysis of protein profile of avirulent promastigotes upon TGF-β exposure. Promastigotes were labelled using \( ^{35} \text{S} \) methionine either at 24°C (lane 1) or heat shocked at 37°C in the presence of 0, 0.1, 0.5, 2 and 5ng/ml of TGF-β (lanes 2, 3, 4, 5 and 6 respectively). Total protein from an equal number of promastigotes was subjected to SDS-PAGE. The gels were stained, destained, dried and finally autoradiographed. The arrow indicates the position of the HSPs 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 two times with reproducible results.
These experiments were repeated more than four times and in our hands TGF-β was found to be ineffective in inducing the stress protein expression of *L. donovani* promastigotes of either virulent or avirulent strains (Fig. 19 and 20).

To examine the effect of IL-4 on the protein synthesis profile of promastigotes of *Leishmania* parasite:
Promastigotes of *L. donovani* were treated with IL-4 at the concentration of 500 U/ml to 1000 U/ml in presence of $^{35}$S-methionine at 37°C for 2h. We did not observe any alteration in the protein synthesis in promastigotes exposed to IL-4 (Fig. 21). Furthermore, no difference was observed in the counts obtained from labeled protein samples in presence or absence of IL-4.

Assessment of promastigotes viability in presence of H$_2$O$_2$:
Motility of promastigotes is known to correlate with their survival. Promastigotes (1x10$^6$ cells/ml) from stationary phase cultures, suspended in the growth medium M199, were incubated with different concentrations of H$_2$O$_2$ for 2h at 37°C. The viability of the promastigotes was monitored microscopically according to flagellar motility by counting a minimum of 200 promastigotes as motile (viable) or non-motile (non-viable). The values are represented as percentage of promastigotes that remained viable at the end of incubation. Increasing concentrations of H$_2$O$_2$ resulted in increasing toxicity as measured by promastigote motility. On exposure to H$_2$O$_2$ upto 60µM the promastigotes of both the strains remained fully viable. Above 60µM
**Figure 21:** Protein synthesis in virulent promastigotes of *L. donovani* in presence of IL-4. Promastigotes were labeled with $^{35}$S methionine at either $24^\circ$C (lane 1) or $37^\circ$C (lane 2) and treated with 0, 500, 1000 U/ml of IL-4 (lanes 2, 3 and 4 respectively). The labelled proteins were separated by SDS-PAGE and autoradiographed. The arrow and 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 two times with reproducible results.

**Figure 22:** Protein synthesis in response to $H_2O_2$ treatment. Promastigote of *L. donovani* virulent (lane A) or avirulent (lane B) strains were exposed to 60μM $H_2O_2$ in presence of $^{35}$S methionine at $37^\circ$C for 2h. Labelled proteins from an equal number of parasite were resolved by SDS-PAGE and autoradiographed. The arrow and 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.
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H₂O₂ the avirulent promastigotes were susceptible to H₂O₂ toxicity in a dose dependent manner. At the end of 2h in the presence of 120µM H₂O₂, only 20% avirulent promastigotes showed motility while 95% virulent promastigotes remained motile. However, H₂O₂, at higher concentrations (above 180µM) was toxic to virulent promastigotes.

**Protein synthesis in presence of hydrogen peroxide:**
Metabolic labelling of parasite was performed by suspending promastigotes in medium containing ³⁵S methionine (at the final concentration of 200 µCi/ml) at 37°C for 2h in presence of H₂O₂. After lysis in SDS buffer, the total cellular protein from an equal number of cells was subjected to SDS-PAGE. Standard molecular weight markers were run each time. Gels were fluorographed, dried and autoradiographed. Protein synthesis in virulent and avirulent promastigotes was examined in the presence of H₂O₂. H₂O₂ treatment led to an appreciable increase in expression of HSP60 in virulent, but not avirulent parasite (Fig. 22). The reduction in total protein synthesis was more marked in avirulent strain.

**Discussion:**
Intracellular replication of *L. donovani* inside macrophage is essential for establishment of the parasite resulting in disease. Since *Leishmania* are exposed to hostile environment throughout their life cycle, HSPs expressed in response to stress encountered in mammalian host may confer protection to the parasite and play a crucial role in their survival. It has been established that subsets of CD4+ T-helper cell Th-1 and Th-2 are endowed with host protective and disease promoting abilities
respectively due to their characteristic cytokine pattern and critical equilibrium between them dictates the outcome of pathogenesis in experimental *Leishmania* infection (Scott, 1990).

*Leishmania* promastigotes and amastigotes can induce the synthesis and release of TNF-α by macrophages. TNF is also essential for maximal expression of cytotoxic levels of nitric oxide which mediates the intracellular killing of *Leishmania* (Green *et al* 1990, 1994). Recent evidences indicate that there may be more than one link between immune mediators and stress proteins (Sugawara *et al*., 1990). It has been shown that the induction of HSPs in tumour cells rendered them resistant to cytotoxicity mediated by TNF (Kusher *et al*., 1990). Furthermore, TNF may itself induce the synthesis of proteins that could protect cells from toxic effects of TNF (Jaattela *et al*., 1992)

TNF treatment led to an overexpression of HSPs 65, 70 and 83 in virulent promastigotes only. Striking differences were observed with respect to HSPs expression between virulent and avirulent promastigotes when exposed to TNF. Unlike virulent parasite, the avirulent promastigotes exhibited remarkably low levels of HSP83. It has been established that TNF induces manganous superoxide dismutase in tumour cells which is essential for cellular resistance to cytotoxicity of TNF. Likewise, by inducing HSPs in *Leishmania* TNF may be activating a protective mechanism against its own toxicity. This hypothesis was supported by our observation that the virulent promastigotes were more resistant to the cytotoxic effect of TNF than the avirulent strain (which failed to overexpress HSPs). That HSPs can
actually protect cells from TNF-mediated toxicity has been clearly shown using antisense HSP70 RNA in tumour (Jaattela et al, 1992).

Phagocytosis of L. donovani promastigotes by human mononuclear phagocytes trigger an oxidative response which results in the generation of a number of toxic oxidant species, including superoxide and H2O2. Heat stress has been shown to increase the resistance of L. donovani chagasi promastigotes against H2O2 toxicity. Furthermore, promastigotes given a pretreatment with actinomycin-D (transcription inhibitor) following heat shock completely abolished this protective effect, thereby suggesting the involvement of HSPs in mediating increased resistance to the toxic effects of H2O2 (Zarlcy et al, 1991). Recently HSPs have also been found to protect tumour cells from oxidative stress (Jaattela et al, 1993).

The virulent promastigotes of Leishmania were found to preferentially synthesize HSP60 upon H2O2 treatment, suggesting the possible involvement of this HSP in overcoming oxidative stress in parasite. As compared to the virulent parasite, the avirulent promastigotes were found to be more susceptible to H2O2-mediated cytotoxicity.

Nitric oxide (NO) has been demonstrated to be the principal effector molecule in the killing of Leishmania by macrophages. The findings that during experimental leishmanial infection, the killing of parasite can be completely reversed by L-NMMA (arginine analogue: blocks NO synthesis) and can proceed normally in a macrophage cell
line deficient in the respiratory burst indicate that NO is both necessary and may be sufficient, to account for the macrophage microbicidal activity (Liew and Cox, 1991).

The preferential synthesis of HSPs 65, 70, 83 by virulent promastigotes and a selective induction of HSP70 only by avirulent parasite when exposed to sub-lethal doses of NO suggests an important role of HSP70 in parasite protection. This view is further supported by the finding of a recent study where HSP70 overexpression was shown to be involved in protecting macrophages against the toxic effects of NO (Hirvonen et al, 1996). Further work in this direction has established that a rat insulinoma cell line stably transfected with human HSP70 gene also confer protection against NO toxicity (Bellmann et al, 1996). HSP70 has also been observed to protect the tumour cells from TNF-mediated toxicity (Kusher et al, 1990).

Invasion of macrophages with bacterial pathogens such as *Salmonella* and *Listeria* also result in induction of HSP70 which is thought to exert protective effect on the host against its own cytotoxic molecules (Kantengwa and Polla, 1993; Schwan and Goebel, 1994). Based on our results we propose a similar role of HSP70 (cell-protection) in *L. donovani* as being observed in other studies.

It has been noted that mRNA specific for HSP83 is increased in late log and stationary phase promastigotes most probably due to nutrient depletion. Interestingly, this correlates with the increased virulence observed in stationary phase promastigotes (Shapira et al,
A differential response by two strains of Leishmania parasite, with significant induction of HSP83 only in virulent strain in presence of TNF and NO indicates that HSP83 along with other HSPs may play an important role in the virulence of Leishmania parasite. The inability of the avirulent promastigotes to mount an effective heat shock response (barely detectable levels of HSP83) upon exposure to TNF or NO further strengthens this view.

Most intriguing is the finding that the effects of NO and TNF on the protein synthesis of L. donovani promastigotes were similar. Both, TNF and NO led to an increased synthesis of HSP 65, 70 and 83 in virulent promastigotes. However, only a selective up-regulation of HSP70 was observed in avirulent parasite. Since TNF has been shown to be essential for the maximal expression of cytotoxic levels of NO, there exists a possibility that the induction of HSPs observed in L. donovani promastigotes upon TNF treatment could actually be due to the production of NO by the parasite. Although, a variety of cell types such as monocytes, macrophages, hepatocytes etc. have been shown to produce NO in presence of appropriate stimulus, it remains to be determined, whether a parasite protozoan such as Leishmania can also release NO when activated by cytokines.

Prior work has demonstrated that NO is involved in the activation of stress protein synthesis. Furthermore, a correlation was observed between heat stress and NO synthesis (Malyshev et al, 1995). Although, the precise mechanism(s) of interrelation between NO and activation of stress proteins is not yet clear, a tentative scheme based on
a number of findings is as follows. Heat shock potentiates generation of free radicals in the body. One intracellular target for free radical is NFkB a factor of transcription of many genes including that coding for inducible NO synthase (iNOS) (Malyshev et al, 1995). Therefore, the increased NO synthase activity following heat shock may be connected with enhanced concentration of free radicals. Another, interesting observation was that a combination of heat shock and NO has been found to have a more pronounced effect on HSPs induction by *L. donovani* virulent and avirulent promastigotes. However, the trigger for increased expression of HSPs could not be determined.

Other cytokines, viz. γIFN, IL-4 and TGF-β also play an important role in pathogenesis of *Leishmania*. γIFN is associated with resistance to *Leishmania* infection where as TGF-β and IL-4 have been linked to susceptibility to the disease. γIFN has been shown to activate macrophages to exert its antileishmanial effects. Also, a direct activity of this cytokine on the growth of extracellular promastigotes has been demonstrated in *in-vitro* (Bhattacharya et al, 1993). In our studies, various concentrations of IFN-γ tested did not alter the synthesis of HSPs by either virulent or avirulent promastigotes of *L. donovani*. IL-4 has also been found to be ineffective in mediating any effect on HSPs expression. Another, very important cytokine produced by activated macrophages i.e TGF-β which has been demonstrated to influence leishmaniasis both *in vivo* and *in vitro* through production of IL-10 (Netto et al, 1992), also was unable to show any significant effect on the stress proteins of *Leishmania*. 
Thus, it appears that all these cytokines influence the process of infection indirectly by either activating or inactivating macrophage functional activities. TNF-α along with the products of oxygen dependent pathway (\( \text{H}_2\text{O}_2 \)) or reactive nitrogen intermediate (NO) has been found to significantly affect HSPs induction in \( L. \text{donovani} \) promastigotes. The overexpression of HSPs by virulent promastigotes in response to various stresses such as heat shock, pH shock, TNF, \( \text{H}_2\text{O}_2 \) and NO in which the avirulent parasites were found to be inefficient indicates that HSPs may play a key role in protecting \( L. \text{donovania} \) parasites against such stressful conditions. Our \textit{in vitro} data suggests that HSPs 60, 65, 70 and 83 synthesized by virulent promastigotes of \( L. \text{donovani} \) may assume importance in overcoming the cytotoxic effects of various stress factors encountered in the mammalian host. Failure of avirulent promastigotes to preferentially synthesize these HSPs may be a critical factor in their inability to survive in the host.