INTRODUCTION AND REVIEW OF LITERATURE
Leishmaniasis constitutes a spectrum of diseases responsible for considerable morbidity and mortality in tropical and sub-tropical areas of the world afflicting an estimated 15 million people. Diverse species of the genus *Leishmania* are responsible for this group of diseases and sandfly either of the genus *Phlebotomous* (old world) or *Lutzomiya* (new world) is invariably the vector that transmits these diseases. Species of *Leishmania* vary in their clinical manifestations ranging from lesions on the skin and mucous membranes, to the lethal visceral form (Table 1). *Leishmania donovani*, the causative agent of kala-azar or visceral leishmaniasis (VL) has also been recognised as an opportunistic pathogen in HIV-infected patients (Trembly *et al*, 1996).

In India, it is believed that kala-azar first appeared in the Gangetic plains of Bengal from where it spread to Assam and Bihar. It retained its endemic form in these areas and later spread to Tripura and Eastern districts of Uttar Pradesh. Sporadic cases of Kala-azar have been reported from the states of Gujarat, Maharashtra, Jammu and Kashmir, Tamil Nadu, Orissa and Himachal Pradesh. Two forms of leishmaniasis viz. Visceral leishmaniasis (VL) or Kala-azar and Cutaneous leishmaniasis (CL) are endemic in the Indian subcontinent.

In India, the worst affected state is Bihar where 33 of the 42 districts are battling the worst ever Kala-azar epidemic, with an estimated 20,000 people having died of VL since 1970. Further, 25,000 to 30,000 people in the state are believed to be infected with the disease. In 1991 alone, 2000 to 3000 people died because of this disease (Katiyar JC, Anuradha, 1994). Leishmaniasis has been identified as a major and
<table>
<thead>
<tr>
<th>Principal species</th>
<th>Type of disease</th>
<th>Site Infection</th>
<th>Main symptoms</th>
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<tr>
<td><em>L. donovani donovani</em></td>
<td>Visceral Leishmaniasis (Kala-azar)</td>
<td>Liver, spleen, bone marrow</td>
<td>Fever, malaise, weight loss, loss of appetite, cough, diarrhoea, anaemia, skin darkening, enlargement of spleen, liver and some times lymph nodes, immuno-suppression, fatal.</td>
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<td><em>L. donovano infantum</em></td>
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<td><em>L. tropica</em></td>
<td>Cutaneous</td>
<td>Skin</td>
<td>Normally self-healing lesions, Although multiple lesion may develop.</td>
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<td><em>L. mexicana</em></td>
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<td><em>L. brasiensis</em></td>
<td>Cutaneous and mucocutaneous</td>
<td>Naso-pharyngeal mucosa</td>
<td>Parasites may spread from untreated dermal lesion to nasopharyngeal mucosa resulting in extensive tissue destruction.</td>
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increasing public health problem by WHO. About 88 countries all over the world are affected by this disease. A recent estimate shows that about 15 million people are infected globally and nearly 40,000 new cases are being reported each year.

**Life cycle:**

*Leishmania* is an obligate intracellular protozoan parasite that leads a digenetic life cycle. The parasite exists in two morphologically distinct forms during its life cycle: the extracellular flagellated form as promastigote in the sandfly vector and an immotile intracellular form as amastigote in the mononuclear phagocytes of the mammalian host (Pearson *et al.*, 1983).

This transformation or conversion from promastigote form to amastigote form involves dramatic morphological changes. Long, slender cells equipped with flagellum are transformed to smaller, ellipsoidal, non-flagellated cells. In addition to the morphological differences found between promastigotes and amastigotes, several molecular and biochemical changes are associated with the transformation event. These include variation in enzymatic activities, differences in proliferation rate, antigenic properties and the pattern of protein synthesis (Chang *et al.*, 1990). It has also been observed that promastigotes undergo additional differentiation both in sandfly vector as well as *in vitro*, from the procyclic to metacyclic forms before finally getting converted into the amastigote form. This process is also associated with changes in parasite virulence (Sacks and Perkins, 1984).
In parasites which cycle between invertebrate and vertebrate hosts, drastic temperature fluctuations are a regular part of their life cycle and induction of stress proteins have been demonstrated in several such organisms including the members of Trypanosomatidae (*Leishmania* and *Trypanosoma*) (van der Ploeg *et al.*, 1985; Dragon *et al.*, 1995), *Plasmodium* (Ardeshir *et al.*, 1987) and *Schistosoma* (Yuckenberg *et al.*, 1987).

At all life stages, *Leishmania* encounter a hostile environment characterized by high hydrolytic and proteolytic activities. When promastigotes are transmitted from sandfly vector to a mammalian host, the parasite experiences a rapid temperature elevation from 22-28°C to 35-37°C. The adaptation of these organisms for survival over a wide temperature range suggests that heat shock responses play a vital role in parasite survival and differentiation. Thus, study of heat shock proteins (HSPs) in parasites such as *Leishmania* is of immense importance, because induction of thermotolerance is a normal physiological phenomenon, that possibly contributes to virulence. However, the molecular events induced in promastigotes due to temperature fluctuations eventually leading to their differentiation are not fully understood.

Heat shock proteins or stress proteins are a ubiquitous, highly conserved family of proteins whose synthesis is increased in response to various types of environmental conditions such as elevated temperature, chemical stress, oxidative injury, inflammation etc. (Lindquist, 1986). The overproduction of HSPs under stress plays an important role in
thermotolerance of the stressed cells. In addition, HSPs also serve vital biochemical functions in normal, unstressed cells.

Role of HSPs in *Leishmania*:

(a) Effect of thermal stress or heat shock:
Exposure of promastigotes of *Leishmania* to elevated temperatures *in vitro* has been shown to result in increased expression of classical heat shock genes and other genes. Multiple protein bands emerge shortly after the heat shock and their number varies with different species. Most of these protein bands appear to be constitutively expressed in promastigotes but become overexpressed in heat shocked promastigotes and amastigotes (Chang *et al*, 1990).

Extracellularly grown promastigotes of *L. mexicana* were found to synthesize increased amounts of proteins of 83, 70, 68, 27, 26, 23 and 22kDa when their growth temperature was shifted from 26°C to 34°C (Hunter *et al*, 1984). In another study, the promastigotes of *L. tropica*, *L. donovani* and *L. enrietti*, when grown at 37°C or 40°C, increased the synthesis of the proteins of 88, 74 and 54kDa (Lawrence and Robert-Gero, 1985). A study on HSPs expressed during *in vitro* transformation of *L. mexicana* from promastigotes to amastigotes showed two different sets of polypeptides synthesized by the transforming parasite. One set of proteins was synthesized at the beginning of the temperature shift and corresponded to the so called HSPs being expressed by promastigotes. The second set of proteins was expressed later and was specifically associated with the amastigote stage.
similar to the patterns of polypeptides synthesized by amastigotes from infected peritoneal macrophages (Alcina and Fresno, 1988).

Most intriguing is the increase in virulence seen with briefly heat-shocked *L. baziliensis panamensis* promastigotes (Smejkal *et al.*, 1988). The molecular changes in these promastigotes accounting for their increased virulence are still an enigma. The overexpressed HSPs are likely to be involved in this phenomenon although their precise functions are yet to be determined. It is not yet clear whether the HSPs are involved in initiating the differentiation of *Leishmania* or are simply required for survival in the host macrophage.

(b) Effect of pH shock:

Temperature in conjunction with pH plays an important role in triggering differentiation of promastigotes to amastigotes and *vice versa* (Zilberstein and Shapira, 1994). *Leishmania* promastigotes, following phagocytosis by host macrophages, are exposed to multiple cellular stresses besides heat shock. Most intriguing is the finding that the formation of phagolysosome complex occurs after leishmanial entry into macrophages and the protozoan transforms to amastigote form and proliferates within the phagolysosomal vesicle (Chang and Dwyer, 1976) implying that *Leishmania* have evolved means for survival in the presence of hydrolytic enzymes and acidic environment of the phagolysosome.

In presence of these stresses the promastigotes successfully differentiate into the amastigote form in order to survive and establish
the infection. Determining the trigger which leads to the transformation of promastigote to amastigote form is potentially of great importance in developing protective strategies.

**Immunity to Leishmania:**
Mammalian cell invasion and intracellular replication by the parasite are essential for establishment of the disease. The first step in the pathogenesis of *Leishmania* involves interaction of promastigotes with the macrophage membrane and its subsequent phagocytosis by the host. Inside the host they are exposed to the toxic oxygen metabolites, proteases, lipid mediators and cytokines of the activated macrophages (Kantengwa and Polla, 1993).

Cytokines are important in regulation of leishmaniasis through activation of macrophage antimicrobial activity. The course of *Leishmania* infection appears to be determined by the pattern of cytokines produced by *Leishmania* reactive CD4+ Th-1 and Th-2 Cells (Coffman et al, 1991). Detection of high levels of IL-4 in the plasma of Kala-azar patients suggests an important role of cytokines in *Leishmania* infection in humans (Zwingenberger et al, 1990).

There is a broad consensus that the principal effector mechanism of murine macrophages for destruction of *Leishmania* is induction of nitric oxide (NO) synthesis from L-arginine by interferon-γ (γ-IFN) and tumor necrosis factor alpha (TNF-α). *Leishmania* promastigotes and amastigotes induce the synthesis and release of TNF-α in macrophages. TNF-α is essential for the production of cytotoxic levels of NO which
mediates the intracellular killing of the parasite (Green et al 1990, 1994). There is a likelihood that the induction of HSPs in response to stress encountered in macrophages may confer protection to parasites and play a crucial role in their survival in the mammalian host.

**Host-parasite Interactions:**
Heat shock proteins (HSPs) occupy an intriguing position in the complex web of host-parasite interactions. Intracellular infection represents one such situation where the pathogenic microorganisms as well as the host cell are under considerable stress (Kantengwa and Polla, 1993).

Members of HSP70 and HSP60 families of microbial origin have been demonstrated to be potent immunogens of pathogenic microorganisms such as *Trypanosoma, Plasmodium, Schistosoma, Leishmania* and *Mycobacterium* (Lathigra et al 1991; Young and Elliot 1989; Young et al 1988). Evidence is fast emerging in favour of the involvement of HSPs as important elements in intracellular pathogenesis of *Leishmania* (Polla 1991; Rey-ladino and Reiner 1993; Salotra et al 1994, 1995).

Expression of parasite derived HSPs in macrophages following infection with *Leishmania* has been noted during *in vitro* experiments. However, no information is yet available about expression of host-derived stress proteins in response to leishmaniasis.
Our endeavour would be to mimic the *in vivo* conditions *in vitro* and examine expression of HSPs so as to develop a better understanding of the nature and extent of HSPs' involvement during *Leishmania donovani* infection.

The work presented in this thesis seeks explanations for the following queries:

I) Role of HSPs in differentiation/survival/virulence of *Leishmania donovani*.

   a) Identification and characterization of HSPs of *L. donovani* in promastigote form of virulent and avirulent strains.

   b) To compare the responses of virulent and avirulent strains of *L. donovani* subjected to heat shock or pH shock.

II) To examine if cytokine-mediated effects involved modulation of HSPs expression of parasite origin in *in vitro*.

III) Whether the host cells stressed by the invading parasite synthesize HSPs and whether and how these HSPs affect host-parasite interactions.

   a) Establishing an *in vitro* model of *Leishmania* infection using murine macrophage-like cell line J774.G8

   b) Studying the kinetics of HSPs expression during the course of *L. donovani* infection.

   c) To determine the differences between virulent and avirulent infection with respect to heat shock protein expression.
This study will provide us with the information regarding the role of HSPs as virulence factors in pathogenesis of kala-azar. This information may further be useful for novel strategies for drug-designing and treatment of the disease.

The present work can be broadly divided into two parts:

Part I: Provides information on the role of stress proteins or HSPs in differentiation/survival/virulence of *L. donovani* virulent and avirulent strains.

Part II: Provides information regarding regulated expression of host-derived HSPs following *L. donovani* infection.
REVIEW OF LITERATURE

Virtually every organism from bacteria to mammals has a set of genes that allows cells of that organism to tolerate damage imposed by various types of environmental stress which otherwise will lead to irreversible injury to the organism (Bond and Schlesinger, 1987). These genes were initially recognized in *Drosophila* embryos as puffs in polytene chromosomes arising very shortly after subjecting the embryos to a heat shock (Ritossa, 1962). Subsequently, the protein products of these genes were identified (Tissieres *et al*, 1974) and they were called heat shock proteins (HSPs). Heat shock proteins represent the most conserved proteins throughout evolution. HSPs play vital biochemical functions in normal cells. They are involved in a variety of cellular processes such as translocation of proteins across membranes, protein conformational changes and the responses to elevated temperature. Overproduction of HSPs under stress seems to provide protection to the cell or organism (Lindquist, 1986; Lindquist and Craig, 1988; Pelham, 1988; Newport *et al*, 1988; Polla, 1991; Hendrick and Hartl, 1993).

HSPs or stress proteins are induced in response to various types of environmental stresses such as chemical stress, oxidative injury, inflammation, viral infections etc. in addition to heat shock (Lindquist, 1986; Lindquist and Craig 1988). On the basis of molecular weight and amino acid sequence homologies the major HSPs are categorized into four distinct families (i) 80-90 kDa (HSP90) (ii) 68-72 kDa (HSP70) (iii) 58-65 kDa (HSP60) and (iv) 15-30 kDa (small HSPs).
An Overview

HSP70:
This is the most highly conserved HSP. In eucaryotes HSP70 genes belong to a multigene family whose members respond to temperature in different ways while in *E. coli* a single gene encoding a 70 kDa protein is present (Lindquist, 1986). Homologs of these proteins occur throughout the eucaryotic cell, in the cytoplasm, nucleus, chloroplast, mitochondria and endoplasmic reticulum (Feige and Polla, 1994). Functional and structural studies of HSP70 have indicated that the molecule contains two major domains; a very highly conserved amino-terminal region which contains ATPase activity and the more divergent carboxy-terminal region which is thought to be important in substrate recognition (Flaherty *et al*, 1990).

The ATPase domain of HSP70 show high structural homology to the ATPase domain of hexokinase and actin. On the other hand the carboxy-terminal or peptide binding domain has been proposed to be similar to MHC class I molecule, however the experimental proof of it is still lacking. Binding of nucleotide by hexokinase foster global changes in the tertiary structure of the protein. ATP binding also changes the conformation of actin and allows its polymerization into a filament. By analogy, it has been proposed that ATP hydrolysis by HSP70 effects a conformational change that it is transmitted to the carboxy-terminal domain. This has actually been found true in case of DnaK as revealed by a change in the pattern of tryptic fragments (Hendrick and Hartl, 1993).
HSP70 of *Leishmania* has been demonstrated to be present in both the promastigote as well as the amastigote forms (Searle *et al.*, 1993; Searle and Smith 1993). HSP70 in *Leishmania* has been found to be an abundant protein present in mitochondria in addition to cytoplasm (Shapira and Pinelli, 1989; Searle and Smith, 1993). Cell fractionation of *Leishmania* parasites metabolically labelled at 26°C and 35°C showed that HSP70 had a basal level of expression at 26°C. Exposure of parasite to 35°C led to an increase in expression of HSP70 concomitant with its redistribution in the cytoplasm as well as in the nucleocytoskeletal fraction. An interesting feature of the 70 kDa chaperones of higher eucaryotes is their rapid relocalization into the nucleus early during heat-stress. However, in a recent study with *L. major* the heat inducible HSP70 was observed not to relocate into the nucleus during a heat shock of promastigotes (Brandau *et al.*, 1995). Indirect immunofluorescence as well as biochemical fractionation revealed that these proteins remained exclusively in the cytosol even under stress. These results argue against previous studies of Shapira and Pinelli (1989) in which a portion of HSP70 was found with nuclear fraction of heat shocked *L. mexicana amazonensis* promomastigotes. Possibly, the HSP70 proteins of New World and Old World *Leishmania* behave differently in this regard.

**HSP90:**

This is the second most highly conserved heat shock protein. All eucaryotic cell produce a prominent heat shock in the range of 83-90 kDa. *Leishmania* contains a 83 kDa protein belonging to HSP90 protein
family which has been identified in both stages of the parasite life cycle (Shapira and Pinelli, 1989). In most cells, the HSP90 protein is abundant at normal temperature and induced by heat, but abundance and inducibility vary in a tissue specific manner (Morange et al, 1984). Since it is also glucose regulated, the tissue specific differences in expression may relate to differences in energy metabolism (Kasambalidles and Lanks 1983). The most interesting property reported for HSP90 is its transient association with retroviral transforming proteins and steroid hormone receptor complexes (Brugge et al, 1981; Oppermann et al, 1981; Schach et al, 1985). Cell fractionation studies indicate that HSP83 of Leishmania is a soluble, cytoplasmic protein and immunocytological localization show diffuse cytoplasmic staining with no concentration in mitochondria or other organelles (Shapira and Pinelli, 1989).

**HSP60:**
Electron microscopic studies reveal that HSP60 proteins are large oligomers having 14 subunits with a characteristic double toroid appearance (Hemmingsen et al, 1988; Hutchison et al, 1989). HSP60 comprises of a subclass of sequence related molecular chaperones including stress inducible and non-inducible members found in bacterial cytosol and in the inner space of mitochondria and chloroplast (Hemmingsen et al, 1988). Highly related HSP60 homologs have not been detected in eucaryotic cytosol or ER, however a protein TCP1, showing weak homology to HSP60 has recently been found in eucaryotic cytosol (Hendrick and Hartl, 1993). The HSP60s in bacteria, mitochondria and chloroplast functionally cooperate with a smaller co-
chaperonin protein of 10 kDa subunit size. In *E. coli* and mitochondria these proteins are stress inducible and are called GroES and HSP10 respectively (Hartman *et al.*, 1992; Lubben *et al.*, 1990). Several studies indicate the presence of immunologically related HSP65 and 67 kDa proteins in *Leishmania* promastigotes (Reyladino and Reiner, 1993; Salotra *et al.*, 1995). Recently, HSP60 from *L. major* has been cloned and sequenced (Rey-Ladino *et al.*, 1997).

**Small HSPs:**

They are a heterogeneous group. Nevertheless, they can be considered homologous on the basis of i) limited sequence relatedness, ii) similarities in predicted protein structure, iii) similarities in intracellular distribution, iv) the tendency to form particles of similar size and structure. In *D. melonogaster* four proteins of this class have been extensively characterized: HSP28, 26, 23, and 22 (Lindquist, 1986). Yeast cells produce a prominent protein of 26kDa (Russnak *et al.*, 1983). The small HSPs are particularly prominent and heterogeneous in plant cells. Intriguingly, the small HSPs of most species show homology to alpha-crystalline lens protein. Small HSPs have been implicated to play a role in the differentiation process (Bond and Schlesinger, 1987).

**Identification of HSPs of Leishmania:**

The discovery of HSPs in *Drosophila* provided impetus for research in this particular area. Subsequently, a number of studies revealed presence of HSPs in organisms as diverse as plants, *Trypanosoma*, yeast, *Escherichia coli*, and mammals etc. *Leishmania* parasite experiences a rapid temperature elevation from 22-28°C to 35-37°C as a part of its
normal development cycle. Survival of *Leishmania* parasite over a wide temperature range suggests that heat shock responses play an important role during parasite life cycle.

Hunter and co-workers in 1984, for the first time described the expression and induction of HSPs in promastigotes of *L. mexicana* in response to elevated temperature. They demonstrated an increased synthesis of proteins of 83, 70, 68, 27, 26, 23 and 22 kDa when the growth temperature of promastigotes was shifted from 26°C to 34°C (Hunter et al, 1984).

Subsequent studies detected the presence of HSPs in several other *Leishmania* species, promastigotes of *L. tropica*, *L. donovani* and *L. enrietti* when grown at 37°C or 40°C, increased the synthesis of three proteins of 88, 74 and 54 kDa (Lawrence and Robert Gero, 1985). Promastigotes of *L. donovani* when exposed to 37°C exhibited an increased expression of putative HSPs of 110, 83, 70, 65, 40 and 12 kDa in virulent parasite. However, the attenuated strain showed increased expression of HSP 83, 70 and 65 only upon being subjected to heat shock (Salotra et al, 1994). A protein of 100 to 110 kDa showing homology to bacterial clpB protein and the yeast HSP 110 (Gottesmann et al, 1990; Parsell et al, 1994) has been identified in promastigotes of several *Leishmania* species following heat shock (Hubel et al, 1995; Salotra et al, 1994). Of particular significance is the identification of low molecular mass HSPs in *Leishmania* since these small HSPs have been recognized to have a role in the process of differentiation (Pinelli and Shapira, 1990; Bond and Schlesinger 1987; Salotra et al, 1994).
Owing to the ubiquity of the heat shock response, the remarkable conservation of heat shock proteins and their constitutive expression in some cases argues against an essential role of these proteins in cell physiology, growth and development. Indeed, HSPs have been shown to act as molecular chaperones (Hendrick and Hartl, 1993), as antigens of microbial pathogens (Shinnick, 1991), as virulence factors (Lathigra et al, 1991) etc.

**Role of HSPs as Molecular Chaperones:**

Protein folding has long been viewed as a spontaneous process. The classical experiments of Anfinsen (1973) using ribonuclease has established that the primary sequence of a protein determines its three dimensional structure. But, it has been calculated statistically that time required for a large polypeptide to sample all possible conformation on its way to the native state would be of the order of age of the universe, hence other cellular factors must be involved. Anfinsen himself had suggested the possibility of existence of "Shuffling enzymes" that can catalyze rate limiting steps in protein folding (Hendrick and Hartl, 1993). These cellular factors or shuffling enzymes are collectively called "Chaperones". The term molecular chaperone was coined by Laskey to illustrate the function of nucleoplasmin in the assembly of nucleosomes.

Presently the "Molecular Chaperones" are defined as proteins that bind to and stabilize an otherwise unstable conformer of another protein by controlled binding and release of the substrate protein thereby facilitating its correct fate *in vivo* be it folding, oligomeric assembly,
transport to a particular subcellular compartment or controlled switching between active or inactive conformations. Molecular chaperones are ubiquitous proteins that have been identified in most of the organisms and cellular compartments like mitochondria, endoplasmic reticulum (ER), golgi bodies, chloroplasts etc. (Hendrick and Hartl, 1993). Strong evidence is accumulating that HSPs are necessary for the acquisition of the native structure of monomeric and oligomeric proteins after their synthesis on ribosomes or after transfer across membranes. Many, but not all, molecular chaperones are HSPs that are also abundant in unstressed cells. The strongest line of evidence suggests that the members of HSP60 and HSP70 families of HSPs can act as chaperones both in vivo and in vitro.

Members of HSP70 family are recognized for their peptide binding functions in protecting nascent protein chain after synthesis, in protein translocation through membranes, protein refolding after denaturation or during protein degradation (Feige and Polla, 1994). Precursor proteins destined to mitochondria and endoplasmic reticulum are maintained competent for post-translational membrane translocation by the action of HSP70s, additional proteins components and cytosolic ATP. HSP70 present in the mitochondrial matrix has been found to bind to partly translocated proteins spanning both outer and inner membranes indicating that the chaperone interact with extended polypeptide chain as it exits the inner membrane. Based on these findings it has been proposed that energy of binding by matrix localized HSP70 may lead to unfolding of precursor parts still outside the outer membrane and may thus provide all or part of the driving force for translocation (Neupert et
The newly imported proteins are transferred to mitochondrial HSP60, thus HSP70 plays a dual role in membrane translocation and subsequent folding of proteins.

Several findings indicate a role of BiP in the folding and oligomeric assembly of proteins imported into the ER for example: i) Studies performed using yeast mutant strains defective in Kar2P gene (homolog of BiP) suggests that Kar2P and its homologs play important role in translocation of proteins (Vogel et al, 1990) ii) furthermore it was noted that yeast cells depeleting for Kar2P leads to accumulation of partially translated secreted proteins (Nguyen et al, 1991), iii) a protein spanning the ER membrane during translocation was found to interact with DnaJ homologous membrane protein Sec63P and to Kar2P (Sanders et al, 1992), iv) following translocation, assembly-defective mutant protein, unassembled protein subunits and misfolded proteins have been observed to form stable complexes with BiP, however the normal proteins on the native folding pathway interacts transiently with BiP.

The best example of HSP70 functioning as a molecular chaperone along with other chaperones is provided by DnaK, a homolog in *E. coli*. Wild and coworkers have demonstrated that DnaK and its partner DnaJ together but neither chaperone alone can promote *in vivo* translocation of alkaline phosphatase precursor across the *E. coli* plasma membrane and that over production of these chaperones can compensate for deletions of the secretory pathway chaperone SecB (Wild et al, 1992). *In vitro* studies on folding of rhodanese (a mitochondrial protein)
have shown that DnaJ potentiates the stabilizing effects of DnaK on unfolded form of the protein (Langer et al, 1992). This would effectively increase the concentration of ADP-DnaK, which by analogy to mammalian HSP70 is expected to bind substrate with higher affinity (Palleros et al, 1991). While the more general functions of DnaK, DnaJ and GrpE appears to be in stabilization of polypeptide chains during de novo folding and under stress conditions (Langer et al, 1992) these three proteins have been known for some time to cooperate in specific processes such initiation of λ DNA replication. The critical step in this process is the dissociation of λ P protein from the replication initiation complex. First, DnaJ binds to the initiation complex targeting DnaK to λ P, which can then be released by DnaK in presence of hydrolyzable ATP. GrpE reduces the amount of DnaK needed for this reaction by ten fold (Hendrick and Hartl, 1993).

A similar cooperation between DnaK and DnaJ has been observed during activation of RepA replication protein of phage P1 (Lindquist, 1992). The uncoating of clathrin coated vesicles by eucaryotic HSP70 provides another example of how the general abilities of HSP70s can be utilized for specific purposes (Rothman and Schmid, 1986). Both in vitro and in vivo experiments demonstrate a role of HSP70 under condition of stress. An excess of DnaK protein present during heat treatment has been shown to protect E. coli RNA polymerase from thermal inactivation in vitro (Skowyra et al, 1990). It has also been observed that the activity of a thermolabile luciferase expressed in E. coli depend upon DnaK ,DnaJ and GrpE . The enzyme
loses its activity at 42°C while at 30°C the enzyme retains its 50% activity in a wild type cell but not in a DnaK or DnaJ mutant strains.

The interaction of luciferase with DnaK and DnaJ has been reproduced in vitro. At 42°C luciferase unfolds and forms a ternary complex with DnaK and DnaJ which is stable in presence of GrpE while the protein aggregates in the absence of chaperones. At 30°C the enzyme regains its activity in a reaction dependent on ATP and all three HSPs. The interaction of luciferase with HSP70 of mammalian cells has been demonstrated to show similar properties, although in this case the participation of DnaJ or GrpE homologues has not yet been demonstrated (Li et al, 1992).

HSP70 has also been implicated to play a role in protein degradation. It has been observed that withdrawal of serum from mammalian cells in culture initiates the degradation of a specific subset of cellular proteins containing a consensus peptide sequence motif (K-F-E-R-Q) by lysosomal proteases (Dice et al, 1986). A 70 kDa protein which is biochemically indistinguishable from mammalian HSP70 i.e. prp73 has been identified as a crucial component in the targeting for the degradation of KFERQ protein (Chiang et al, 1989). Furthermore, in E. coli, abnormal proteins that are degraded rapidly by La proteases bind DnaK and GrpE. Therefore it appears that cooperation between the in vitro folding pathway and the protein degradation system would provide effective control over the level of abnormal proteins in the cell (Sherman and Goldberg, 1991).
A number of findings indicate that chaperonin 60s are involved in folding and assembly of proteins. The first evidence that protein assembly in general might require cellular factors came from the study of mif4 mutant yeast which harbours a temperature sensitive mitochondrial HSP60. Although, in these yeasts, import of proteins into the organelle occur normally, newly imported proteins including HSP60 itself failed to assemble (Cheng et al, 1989). E. coli mutants defective in GroEL were found to be unable to assemble the head structure of phage λ and tail assemblies of phage T5 (Georgopoulous et al, 1973; Zweig and Cummings, 1973). A fusion protein containing a cleavable targeting signal joined to the enzyme dihydrofolate reductase (DHFR) was found associated with HSP60 in a protease sensitive, unfolded state upon transport into ATP depleted mitochondria. The HSP60 bound DHFR was chased to a folded, protease resistant form upon incubation with Mg-ATP (Ostermann et al, 1989).

HSP60 also plays vital role in cells under stress as has been shown that over production of GroEL and GroES in bacterial cells lacking a normal heat shock response protects intracellular proteins from aggregating into insoluble complexes (Gragerov et al, 1992). Similarly mitochondrial HSP60 forms complexes with a number of organellar proteins and prevent in vivo thermal inactivation of DHFR imported into the mitochondrial matrix (Martin et al, 1992). It has been further noticed that mitochondrial rhodanese in a binary complex with GroEL is protected from thermal inactivation at 48°C and can be released in active form upon addition of GroES and ATP at 25°C (Mendoza et al, 1992).
Evidence is accumulating in favour of involvement of HSP90 as a molecular chaperone that regulates protein functions. It was observed that glucocorticoid receptors synthesized in vitro in a reticulocyte lysate becomes a part of a large complex containing HSP90 (Catelli et al, 1985). HSP90 functions by binding to receptor such that it is stabilized in a state having high affinity for hormone but cannot bind DNA (Pratt, 1990). Activation of HSP90 bound receptor by addition of ligand is an ATP requiring process that involves binding of ligand, release from HSP90 and changes in receptor conformation to a form that has high affinity for DNA (Pratt, 1990). It has been demonstrated that expression of glucocorticoid receptor in yeast strain depleted for HSP90 leads to synthesis of receptors that do not stimulate transcription of an induced receptor responsive gene (Picard et al, 1990). HSP90 has been shown to bind to pp60v-src and inhibits its tyrosine kinase activity. On the other hand its binding to casein kinase II has been shown to stimulate the activity and the protein has been found to be less prone to aggregation (Miyata and Yahara, 1992). HSP90 is proposed both to inactivate and to foster transport of the oncogene products (Oppermann et al, 1981).

**HSPs as antigens of microbial pathogens:**

The first clue as to the immunoreactivity of HSPs came from the work of Bianco et al (1986) showed that the amino acid sequence of a major antigen of the malarial parasite had 70% identity to the sequence of a 70-kDa HSP of *Drosophila*. Interest in this field was further stimulated by reports describing high homology between immunoreactive 65-kDa
protein of *Mycobacteria* and a 60-kDa heat shock protein from *E. coli* (Shinnick *et al.*, 1988; Thole *et al.*, 1988; Young *et al.*, 1988).

Members of the HSP70 family have been shown to be antigens of several parasites and bacteria. Dubois *et al.* (1984) have demonstrated that antibodies to 72-kDa protein (a member of HSP70 gene family) of *Plasmodium falciparum* leads to immune protection in squirrel monkeys. The genes or portion of the genes for at least four members of *P. falciparum* HSP70 family have been cloned and sequenced (Bianco *et al.*, 1986; Ardeshir *et al.*, 1987; Kumar *et al.*, 1988; Mattei *et al.*, 1988; Peterson *et al.*, 1988; Kun and Mullerhill, 1989). It was observed that antibodies from pooled human sera affinity purified using HSP70 from two different recombinant clones did not cross react with the other protein suggesting that the immune response to malarial HSP70 is predominantly directed towards nonconserved epitopes (Peterson *et al.*, 1988). Virtually all sera from humans and animals infected with *Schistosoma mansoni* was found to react with a 70-kDa antigen (Hedstrom *et al.*, 1987, 1988). Furthermore, antibodies to this protein have been shown to correlate with immune protection in mouse model. Several lines of evidence suggests that the immune responses to the 70-kDa antigen is mainly directed toward nonconserved sequences (Hedstrom *et al.*, 1988; Scallon *et al.*, 1987).

Most individuals infected with *Brugia malayi* produce antibodies reactive with a 70-kDa antigen (Selkirk *et al.*, 1989). The gene encoding this protein has been isolated from a *B. pahangi* cDNA library and shown to be a part of a multigene family. The cloned protein was found
to show homology to members of HSP70 family, particularly a rat HSP70 (85% amino acid sequence identity). The majority of the immune response to this protein in infected persons seems to be directed against epitope found specifically on the *Brugia* protein. However, reports showing cross-reactivity of sera from some infected individuals with molecules of HSP70 from *P. falciparum* and *S. mansoni* also exist (Selkrik, 1989).

HSP70 has also been shown to be a major target of humoral immune response during *Leishmania donovani* infection (Macfarlane *et al*, 1990). Serological analysis of recombinant HSP70, expressed by a series of deletion constructs identified the carboxy-terminal region as the immunodominant site (Wallace *et al*, 1992). Recombinant *Leishmania* HSP70 and HSP90 proteins were recognized by sera from kala-azar patients but not Chagas disease patients despite more than 80% identity between *Leishmania* and *T. cruzi* sequences. This specificity could have arisen because the HSP fusion proteins used as target antigens contained C-terminal sequences which comprises the most divergent part of the molecule (DE Andrade *et al*, 1992).

An immunoreactive 70-kDa antigen has also been identified in *Mycobacterium leprae, M. tuberculosis, M. bovis BCG* (Britton *et al*, 1986; Watson, 1989). A 75-kDa protein which shows homology with *E. coli* DnaK protein and eucaryotic HSP70 has been recognized in *Chlamydia trachomatis*. Serum antibody levels to this protein have been correlated with protection against ascending-fallopian tube infection (Brunham *et al*, 1987). Further studies revealed that this protein appears
to be surface accessible and monospecific antibodies directed against it can neutralize infectivity (Maclean et al., 1988; Daniliton et al., 1990). Here again, the immune response has been shown to be primarily directed at non-conserved epitopes (Birkelund et al., 1989).

Two set of studies on different groups of bacteria led to realization, that a prominent bacterial antigen was actually a heat shock protein. Independent studies by Kaijser (1975) and Hoiby (1975) identified a 60-kDa protein as widely cross-reactive antigen of *E. coli* and of *Pseudomonas aeruginosa* which was named as common antigen. Subsequent studies showed that proteins cross-reactive with common antigen are present in virtually all bacteria. It was further shown that the highly immuno-reactive 65-kDa protein of *M. tuberculosis* and *M. leprae* are the mycobacterial counterparts to common antigen and these antigens are homologous to the *E. coli* GroEL heat shock protein (Shinnick et al. 1988; Thole et al., 1988; Young et al., 1988). Several line of evidence indicate that HSP60 of *Mycobacteria* is a immunodominant antigen. There is a broad consensus that the humoral and cellular immune responses to HSP60 is directed against both conserved and nonconserved epitopes (Mehra et al., 1986; Munk et al., 1988; Thole et al., 1988; Anderson et al., 1988; Shinnick et al. 1988; Lamb et al., 1989). Immunoreactive HSP60 homologues have been identified in species of *Borrelia* (Hansen et al., 1988), *Chlamydia* (Morrison et al., 1989), *Coxiella* (Vodkin and Williams 1988), *Salmonella* (Buchmeire and Heffron, 1990) etc. HSP60 has recently been recognized as a strong immunogen in case of parasitic infections.
HSP60 displays a protective efficacy against toxoplasmosis (Nagasawa et al, 1992) and Histoplasmosis (Gomez et al, 1995).

In a recent study, purified recombinant HSP60 from *L. major* was used to examine sera from patients of leishmaniasis for presence of antibodies to HSP60. Unlike uninfected controls, sera from patients reacted strongly with recombinant *leishmania* HSP60 (Rey-Ladino et al, 1997). Following infection of macrophages with *Leishmania donovani*, the expression of 65 and 67-kDa, HSP60 related proteins of parasite origin was observed suggesting that during *in vivo* infection these molecules may be expressed at high levels and become important immunogens (Reyladino and Reiner, 1993).

The HSP90 proteins of parasites viz *Trypanosoma cruzi*, *S. mansoni*, *P. falciparum*, *Leishmania* have been reported to be immunogenic. Dragon et al (1987) isolated a clone from a cDNA library of *T. cruzi* using Chagas’ disease patient sera that encode a part of 85 kDa protein. Sequence analysis of this cDNA clone and parts of clone containing the genomic copy of this gene revealed that the *T. cruzi* protein had 60-65% identity with corresponding HSP83 of *Drosophila* and HSP90 of yeast. A strong humoral response against Leishmania recombinant HSP90 was observed during visceral leishmaniasis. This HSP was found to be specifically recognized by antibodies present in sera from kala-azar patients but not Chagas’ disease patients inspite of greater than 80% sequence identity between *T. cruzi* and Leishmania HSP90. The recombinant HSP90 used in ELISA assays during this
study was demonstrated to contain sequences from C-terminal region of the molecule which is the most divergent part (DE Andrade et al, 1992).

**HSPs in differentiation and virulence of microorganisms:**

Many dimorphic pathogens including *Leishmania* encounter dramatic changes in ambient temperature during their complex life cycle. There is substantial evidence to indicate that in eucaryotic parasites the expression of HSPs in response to temperature shifts is an early event of co-ordinated programme of gene expression leading to the differentiation of these parasites (van der Ploeg et al, 1985; Newport et al, 1988). A study on HSPs expression during *in vitro* transformation of *L. mexicana* from promastigotes to amastigotes described the synthesis of two different sets of polypeptide by transforming parasites. One set of proteins was synthesized at the beginning of the temperature shift and corresponded to the so called HSPs being expressed by promastigotes. The second set of proteins was expressed later on and was specifically associated with the amastigote stage, similar to the pattern of polypeptides synthesized by amastigote from infected peritoneal macrophages (Alcina and Fresno, 1988). These results suggest that HSPs may play a function at a defined time in the transformation of the parasite. In *L. braziliensis* an increase in HSP production has been correlated with an increase in infectivity associated with differentiation from promastigote to amastigote form (Smejkal et al, 1988).

Searle *et al* (1989) observed differential expression of HSP70 gene of *L. major* during differentiation of parasite from noninfective procyclic to infective metacyclic forms even in the absence of a
temperature shift. This mimics the situation in vivo where differentiation to the infective (virulent) form occurs in response to nutritional deprivation in the insect hind gut just before a next blood meal. This differentiation has been shown to be accompanied by changes in HSP83 levels (Shapira et al, 1988). However, the involvement of HSPs in pre-adapting the parasites may not be a general phenomenon since malarial sporozoite do not express HSP70 even though all the blood stages do (Bianco et al, 1986).

Role of HSPs during Infection:
Numerous investigations on the role of HSPs during host-pathogen interaction have revealed HSPs as important element in pathogenesis of several intra-cellular infections (Engman et al, 1990; Hedstrom et al, 1987; Kaufmann, 1990; Kumar et al, 1990). Infection represents particularly interesting situations, since invading pathogens have the potential not only to induce HSP expression by host cells but also to elicit immune responses to their own HSPs (Kaufmann 1990; Young 1990; Maresca and Carratu, 1992).

Phagocytosis of S. aureus by human macrophages has been demonstrated to induce in these cells the co-ordinate upregulation of HSP70, hemeoxygenase and superoxide dismutase which was proposed to provide protection to macrophages against the toxic factors released during phagocytosis (Kantengwa and Polla, 1993). A role of HSPs in the immuno-pathology associated with leprosy has been suggested during infection of schwann cells with Mycobacterium leprae (Steinhoff et al, 1988; Mistry et al, 1992). An increase in mRNA levels of host
HSP70 and 90 were observed in J774 macrophages in response to *Listeria* infection while the level of HSP60 mRNA remained unaltered. Further analysis revealed that HSP90 and PTP (protein tyrosine phosphatase) were induced only during infection with wild type *L. monocytogenes*. The kinetics of HSPs induction at specific stages of intracellular *Listeria* infection indicates that subversion of signal transduction routes in the host cell may be critical factor for intracellular survival of this bacteria (Schwan and Goebel, 1994).

Induction of HSP70 in virulent strains of *Toxoplasma gondii* as a consequence of immunological stress experienced in immuno-competent host has been suggested to provide protection to the virulent parasite there by enabling it to persist and multiply in host macrophages while avirulent strains were either forced to develop into cyst or destroyed by the host's immune response (Lyons and Johnsons, 1995). A few recent investigations on the expression of parasite derived HSPs during leishmaniasis suggests that during *in vivo* infections, HSPs may be expressed at high levels and become important immunogens. It was observed that *L. major* synthesized high levels of its own stress proteins in response to infection of human and murine macrophages (Kantengwa *et al*, 1995). A rapid and co-ordinated expression of heat regulated 65 and 67-kDa proteins of parasite origin has been demonstrated following infection of murine macrophages with *L. donovani* (Reyladino and Reiner, 1993). Recently Streit *et al* (1996) assessed the expression of HSP70 and 90 of *L. chagasi* following intracellular conversion of promastigotes to amstigotes in human macrophage-like cell line. The amount of both HSP70 and 90 increased transiently after phagocytosis
and subsequently decreased to base line levels. The relatively high level of expression of *Leishmania* HSPs upon infection suggests that these proteins are involved in pathogenesis and may serve as important targets of immune response.

**HSPs in response to stresses other than heat shock:**

HSPs/Stress proteins are known to be induced by a wide variety of stimuli such as heavy metal ions, sodium arsenite treatment, reactive oxygen intermediates, nutrient limitation etc. besides heat shock.

Of particular importance is the oxidative stress associated with exposure to phagocytic cells of the host immune system (Hasset and Cohen, 1989). Microorganisms present in inflammatory foci and those pathogens which enter phagocytes are exposed to a variety of reactive oxygen metabolites and their ability to survive such exposure may play an important role in the infective process. During adaptation to hydrogen peroxide 30 proteins were induced, including HSPs such as DnaK chaperones by *S. typhimurium* (Christman *et al*, 1985; Morgan *et al*, 1986). A group of nine proteins including DnaK which were coordinately regulated by the oxyR gene product were studied extensively. Mutant strains of *S. typhimurium* constitutively synthesizing the oxyR regulated genes were found to be resistant to killing by hydrogen peroxide while strains unable to induce the oxyR regulon had enhanced hydrogen peroxide sensitivity. In another study, exposure of *E. coli* to superoxide radicals has been investigated using redox cycling reagents and while the response overlaps to some extent with oxyR, the GroE HSPs have been implicated in this form of
oxidative stress (Walkup and Kogoma, 1989; Greenberg and Demple, 1989). Synthesis of another HSP, HtpG or C62.5 the bacterial equivalent of the eucaryotic 90 kDa, HSP90 family (Dardwell and Craig, 1987) is also induced by superoxide radicals (Greenberg and Demple, 1989).

Promastigotes of *Leishmania* heat pretreated and then exposed to hydrogen peroxide has been found to show increased resistance to hydrogen peroxide mediated toxicity. However, magnitude of protection against hydrogen peroxide toxicity after heat shock differed significantly between log and stationary cultures of promastigotes. The mechanism underlying the resistance to hydrogen peroxide toxicity of promastigotes after heat shock seems to involve production of protein(s) presumably HSPs with protective function (Zarley *et al*., 1991). In *in vitro* experimental system, promastigotes of *L. donovani* has indeed been shown to increase the expression of HSP60 in addition to HSP70 and 83 in response to hydrogen peroxide. The induction of HSPs by hydrogen peroxide stress was observed in virulent parasites but was lacking in the avirulent promastigotes (Salotra *et al*., 1995).

Nutrient limitation also represents a kind of stress that may contribute to appearance of stress proteins. Promastigotes of *L. major* subjected to nutritional stress (serum deprivation *in vitro*) responded by increase in relative synthesis of putative HSPs 82, 70 and of proteins of 79 and 41 kDa but decrease in synthesis of proteins of 38 and 28 kDa (Toye and Remold, 1989). It was noted that mRNA specific for HSP83 is increased in late log and stationary phase promastigotes, most
probably due to nutrient depletion. Interestingly, this correlated with the increase in virulence observed in stationary phase promastigotes (Shapira et al, 1988).

A high proton concentration within the phagolysosomal compartment poses another stress for pathogenic parasites such as *Leishmania*. A significant increase in HSPs with a concomitant decrease in total protein synthesis was observed in promastigotes of *L. donovani* upon being subjected to a low pH. Virulent promastigotes showed a significantly greater increase in the relative expression of the major HSPs (HSP83 and 70) as compared to attenuated promastigotes. Further, a combination of low pH and heat shock led to an increase in relative expression of both HSPs 83 and 70 in virulent parasites while only HSP70 in the avirulent strains (Salotra et al, 1994).

**Cytokines during infection:**
A major advance in immunology was the demonstration that CD4⁺ Th cells are composed of subsets based upon the cytokines produced after stimulation, and that these distinct T cell subsets often influence the outcome of infection (Mosmann et al, 1986). CD4⁺ Th1 cells produce IL-2 and γIFN, primarily mediate cell mediated immunity and are often associated with bacterial, Protozoan, and viral infections (Mosmann et al, 1986). In contrast, Th2 cells produce IL-4, IL-5, and IL-10, mediate humoral and allergic responses and often associated with helminthic infections (Mosmann et al 1986). Observation from several *in vitro* and *in vitro* models indicate that the cytokines present during primary
antigens stimulation are a major factor in determining the differentiation pathway of naive T cells (Coffman et al., 1991; Scott, 1991).

Cytokines play an important role in the regulation of leishmaniasis through activation or inhibition of macrophage antimicrobial activity. In murine experimental leishmaniasis a close correlation has been observed between the outcome of infection and the type of T-cell response to the parasite. 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, γIFN and lymphotoxin, the animal succeed in eliminating the parasite and controlling the infection (Scott, 1990). A number of cytokines such as IL-4, γIFN, TGF-β, IL-10, IL-12, TNF-α etc. have been reported to be directly or indirectly involved in determining the outcome of the Leishmania infection (Scott, 1990; Green et al., 1990; Barral et al., 1992; Netto et al., 1993; Bhattacharya et al., 1993).

The notion that cytokines may play a role in regulating the infection in humans is supported by the finding of elevated IL-4 levels in kala-azar patients sera (Zwingenberger et al., 1990) and by promising results of including γIFN in the treatment of human visceral leishmaniasis (Badaro et al., 1990). Furthermore, L. donovani reactive Th1 and Th2 like cells have been identified in individuals who had recovered from kala-azar indicating that humans also have the potential for both Th1 and Th2 responses to Leishmania infection.
In murine models, it has been demonstrated that the outcome of infection with *L. major* depends on the local cytokine environment at the time of inoculation of parasite similar mechanisms are likely to be operative in humans. In a recent study with *L. major* it has been demonstrated that the *Leishmania* parasite induces the production of two competing cytokines.

1) The TNF (parasite destruction) (2) or TGF-β (parasite survival) and the presence as well as quantity of γIFN at the time of infection has been shown to be crucial for *Leishmania* pathogenesis. A significant body of literature suggests that nitric oxide (NO) derived from L-arginine is the essential molecule mediating killing of promastigotes and amastigotes of *Leishmania*. Cytokines have been implicated to have a role in the regulation of NO production by *Leishmania* infected macrophages. TNF-β and γIFN have been shown to be essential for the production of cytotoxic levels of NO in *Leishmania* activated macrophages.

Evidence is growing in favour of a close association between stress proteins/heat shock proteins and NO. NO induced following heat shock has been shown to trigger the activation of stress genes in several mammalian organs (Malyshev *et al*, 1995) On the other hand, stress proteins have been demonstrated to confer protection to macrophages (Hirvonen *et al*, 1996), rat islet cells (Bellmann *et al*, 1995) against the toxic effects of NO.
Structure, Organization and Regulation of HSP genes in *Leishmania*:

Heat shock genes provide a suitable system to study temperature induced gene regulation in digenetic parasites such as *Leishmania*. Several heat shock genes from parasites have been cloned and sequenced (Maresca and Carratu, 1992). However, most of the studies have been done at structural level and not at transcriptional level, so that very little is known about the mechanism of heat-shock gene regulation in parasites.

The sequence and genomic organization of hsp70 and hsp83 genes has been examined in various species of *Leishmania*. In *L. major* two low-copy number sequences with homology to eucaryotic hsp70 genes have been identified on different chromosomes (Searle *et al*, 1989) in addition to a tandemly repeated hsp70 gene family (Lee *et al*, 1988) that maps to a third distinct chromosome (presumably homologous to chromosome 17 of the *L. major* karyotype (Samaras and Spithill, 1987). A cognate member of hsp70 gene has been cloned from an expression library of *L. donovani* by screening with serum from patients with visceral leishmaniasis. It was found to be present in the genome on a single chromosome as a series of approximately twelve 3.7Kb direct tandem repeats (Macfarlane *et al*, 1990). Thus the HSP70 related genes could occupy almost 5% of the chromosome. The corresponding mRNA and protein for this gene are both constitutively expressed in promastigotes as well as amastigotes.
Genomic organization of hsp70 genes in *L. amazonensis* is quite similar to that in *L. major*. Seven hsp70 genes are organized into a 2.4Kb gene locus containing 3.5 Kb tandem repeat. An eighth hsp70 sequence is located at a distant site (Bock and Langer, 1993). A sequence comparison of the cDNAs with the genomic clone identified the following features in hsp70 gene of *L. amazonensis*: a pyrimidine-rich region located upstream of the splice acceptor site, a 143-nucleotide (ntd) 5'-Untranslated region (UTR) region, a 1959 ntd coding region, 3'-UTR sequences which contained G/C-rich areas and stretches of TG or CA dinucleotides. A eucaryotic polyA addition site consensus signal was absent as in other trypanosomatid genes analyzed. The CGCAC/G motif observed in the vicinity of splice acceptor sites in several *Leishmania* genes (Kalper et al, 1990) was also present in the *L. amazonensis* hsp70 gene.

Studies of Lee et al (1988) revealed presence of four hsp70 genes arranged in tandem repeats in *L. major*. These are 89% conserved at the amino acid level and 80% conserved at the nucleotide level, when compared to *T. brucei* hsp70 genes. The codon usage of the *L. major* genes favours G/C bases. The extreme C-terminal coding regions of hsp70 genes are more diverged than N-terminal regions. The expression of these four hsp70 genes was found to be increased *in vivo* and *in vitro* in response to a temperature shift from 25°C to 37°C. In contrast, the high rate of transcription of a fifth hsp70 gene, located at a separate locus, was unaffected by temperature shifts. The hsp70 mRNA was found to have mini-exons transspliced onto the 5'ends that shared unusually long untranslated extensions containing repetitive sequences.
Since each of the hsp70 genes identified was expressed normally in *L. major* substocks that lost the capability to differentiate in response to an *in vitro* temperature shift, the inability to differentiate did not result from a general defect in the temperature-dependent control of transcription.

How the hsp70 genes of *Leishmania* are regulated during development is of considerable interest in itself, but in addition, there is a real need to understand differential expression of this gene family because of high immunogenicity of some HSPs in both *Leishmania* and other parasitic species. In a subsequent study with *L. major* two low-copy number sequences with homology to eucaryotic hsp70 genes were identified on separate chromosome by Searle et al (1989). Both these genes were later sequenced and found to share sequence identity with members of eucaryotic HSP70 family (Searle et al, 1993; Searle and Smith, 1993). Their protein products were found to be constitutively expressed at all stage of parasite life cycle, but their intracellular distribution was different. While one was localized to the cytoplasm, the other HSP70 was localized to the mitochondrion. It had an N-terminal sequence characteristic of a mitochondrial targeting signal.

In both mitochondrial and cytosolic HSP70, the deduced open reading frame showed features common to HSP70 stress proteins including ATP-binding domain and a putative calmodulin-binding domain (Stevenson and Calderwood, 1990, Hightower, 1991), suggesting that leishmanial HSP70 is capable of binding ATP and may be regulated by calmodulin. There is an extremely high degree of
Figure 1: Comparision of the C-terminal amino acid sequences beginning at position 252 of Hsp90s from *L. donovani*, *L. mexicana* and *T. cruzi*. Divergence in sequence homology is marked with the altered amino acid (33).
similarity between different species of *Leishmania* (95%) and moderate sequence identity with *Trypanosoma* (85%) and human HSP70 (70%). The C terminal regions are observed to be the least conserved.

The gene of hsp83 in *L. mexicana amazonensis*, cloned from a genomic library, was shown to be present in at least four tandemly arranged repeats of 4 Kb units (Shapira and Pinelli, 1989). Sequence analysis of the hsp83 repetitive unit revealed a high degree of conservation with the coding sequence of *T. cruzi*. Analysis of codon usage showed that the third position was preferentially occupied by G/C bases in both *Leishmania* and *T. cruzi*, but *Leishmania* codon usage was much more biased to G/C (Shapira and Pedraza, 1990). The hsp83 mRNA included untranslated extensions of 320 bases upstream to the coding gene, and 900 bases downstream to it (Aly et al, 1993). Comparison of the deduced C-terminal 453 amino acids of hsp83 of *L. donovani* with sequence of *L. mexicana amazonensis* and *T. cruzi* is shown in Fig 1.

The two *Leishmania* proteins are identical at 93% of positions while the *T. cruzi* protein has 85% identity with *L. mexicana amazonensis* and 82% identity with *L. donovani* protein. The C-terminal regions are observed to be the most divergent, similar to the trend for hsp70 across species. Recently, hsp100 gene has been identified in *L. major* as a single copy per haploid genome (Hubel et al, 1995). Structural analysis revealed a remarkable sequence conservation to the bacterial clpB protein and the yeast 104-kDa HSP (Gottesman et al,

Regulation of stress-induced genes has been thoroughly studied in eucaryotes, providing a basis for comparison with *Leishmania*. In most non protozoan eucaryotes expression of heat-shock genes is regulated at transcriptional level. Heat shock leads to interaction between the Heat Shock Factor (HSF) and a consensus DNA sequence denoted by Heat Shock Element (HSE) which is located upstream from TATA box. Heat shock in Hela and *Drosophila* cells results in binding of the HSF to the HSE, or in yeast, to the phosphorylation of an already bound HSF. In all these non-protozoan organisms, the DNA-protein interaction following heat-shock results in activation of stress gene transcription.

A recent study concluded that the expression of hsp83 gene in *Leishmania* is regulated post-transcriptionally (Argaman *et al*, 1994). Transcripts of hsp83 accumulated upon temperature elevation, however, in contrast to non-protozoan eucaryotes, no transcriptional activation was observed. The increase in the hsp83 mRNA level evolved from temperature induced variations in mRNA turnover the hsp83 transcript was rapidly degraded at normal temperatures, whereas heat-shock led to its stabilization. The quick decay of mRNA at lower temperatures was dependent on active protein synthesis suggesting that it may be controlled by a labile nuclease. The increase in temperature also affected translational regulation of HSPs and synthesis of hsp83 was more efficient at 35°C than at 26°C. However, the effect on translation was transient and the steady state level of the protein was hardly altered.
Previous attempts to identify HSEs within the intergenic region sequences of heat shock genes in trypanosomatids revealed DNA elements with partial homologies, but assays of their function were not performed. Comparison of hsp83 intergenic sequences from *L. mexicana amazonensis* with the published hsp83 upstream sequences of *T. cruzi* and full intergenic region of *T. brucei* did not reveal any striking homologies or consensus sequences. A similar approach using the intergenic region of HSP70 from *L. major* and *T. brucei* did not reveal any sequences of homology, except for a poly-pyrimidino stretch located 50bp upstream to the mini exon acceptor site (Lee *et al.*, 1988, Glass *et al.*, 1986). Further, studies are required to define the components involved in mRNA stabilization of *Leishmania* and to examine whether they are unique for heat shock genes or common to other genes as well.

The genome organization of kinetoplastid organisms is unique among eucaryotes. Most structural genes are arranged in direct tandem repeats and transcribed into polycistronic RNAs that are further processed by polyadenylation and trans-splicing (Agabian, 1990). RNA transcription initiation sites are lost from mRNAs during the process of maturation and there are still very few data on the nature of the transcriptional promoter elements. A few promoters were recently characterized in *T. brucei* showing Poll features (Zomerdijk *et al.*, 1990), however, they were not active in *Leishmania* parasites and showed no homologies to any promoter elements from higher eucaryotes. No promoter has yet been located or identified for any of the *Leishmania*
structural genes. Comparison of intergenic sequences of genes that are expressed under similar conditions (such as HSPs) may reveal shared sequences with a putative role in control of gene expression.

The foregoing view of literature suggests that heat shock proteins (HSPs) are involved in a variety of cellular processes and are particularly important in the fields of immunology and infectious diseases.

The parasites of the genus *Leishmania* shuttle between the insect vector and mammalian host during their life-cycle and hence are exposed to change in environmental conditions viz. temperature and pH. A significant body of literature suggests that promastigotes of *Leishmania* upon being subjected to heat shock show increased synthesis of several HSPs which may have a role in the differentiation/virulence of the parasite (Hunter *et al.*, 1984; Lawrence and Robert-Gero, 1985; Smejkal *et al.*, 1988; Brandau *et al.*, 1995; Hubel *et al.*, 1995). Salotra *et al.* (1994) have further demonstrated differential expression of HSPs by virulent and attenuated strains of *Leishmania* in response to heat shock or pH shock.

The role of cytokines is well documented and distinct patterns of cytokines have been associated with the susceptibility or resistance to the leishmanial infection. Cytokines influence the course of infection by either activation or suppression of macrophage functional activities. A direct effect of γIFN and TNF-α has also been observed on the growth of promastigotes of *Leishmania* (Bhattacharya *et al.*, 1993).
Earlier investigations have revealed an induction of major HSPs of parasite origin in *Leishmania* infected macrophages (Kantengwa and Polla, 1993; Reyladino and Reiner, 1993; Kantengwa *et al.*, 1995; Streit *et al.*, 1995). However, a lack of host stress response during leishmaniasis has been observed by earlier workers.

A shift in temperature is an integral part of *Leishmania* life-cycle, keeping this in view attempts were made to identify and characterize HSPs of *L. donovani* during the course of present study. Furthermore, we have assessed if cytokines mediated effects involved modulation of HSPs of parasite origin. The stress response in *Leishmania* infected macrophages was characterized immunologically using host specific antibodies.