

**DYNAMICS OF STRESS PROTEIN EXPRESSION IN LEISHMANIA-INFECTED  
MACROPHAGES: EVIDENCE FOR SELECTIVE INDUCTION AND RECOGNITION  
OF LEISHMANIA HEAT SHOCK PROTEIN 60**

**By**

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## ABSTRACT

Heat shock protein (HSP) expression was examined in murine bone marrow-derived macrophages infected with stationary phase promastigotes of *Leishmania donovani*. Immunoblotting with a rabbit polyclonal antiserum raised against HSP60 from *Heliothis virescens* (moth), revealed the *de novo* appearance of proteins with subunit sizes of  $M_r$  65,000 and 67,000 in leishmania infected macrophages. Expression of the novel  $M_r$  65,000 and 67,000 proteins in infected cells was coordinately regulated and at 24 hr of infection reached maximal levels of 52-100% increases above initial levels at 3 hr. Proteins with identical electrophoretic mobilities and which were similarly regulated in response to heat were also detected in leishmania promastigotes. The appearance of these proteins in macrophages was specific to leishmania infection in that neither protein was detected in non-infected cells either in the basal state or following several treatments including: (i) infection with *Y. pseudotuberculosis*, (ii) phagocytosis of *S. aureus*, (iii)  $\text{NaAsO}_2$ , or (iv) heat shock.

A monoclonal antibody that recognizes both mammalian HSP70 and HSP70 from *Plasmodia* detected single isoforms of both leishmania and murine HSP70 in infected cells and neither protein changed quantitatively during infection. Levels of host heat shock proteins 60, 70 and 90 did not change in response to infection, however, marked induction of the mammalian stress protein heme oxygenase-1 was observed under these conditions. Further evidence for selective expression of the  $M_r$  65,000 and 67,000 heat-regulated leishmania proteins was provided by the finding that as their concentration was increasing, the abundance of the leishmania surface protease gp63 in infected cells was noted to decrease.

The prominence of leishmania HSP60 within infected macrophages suggested the possibility that it might be a target of the host response. To facilitate further studies concerned with the role of this protein in the host response and in the pathogenesis of the leishmaniases, the *HSP60* gene of *Leishmania major* was cloned, sequenced and expressed. A  $\lambda$ EMBL-3 *L. major* genomic library was screened with a PCR-generated DNA probe derived from a highly conserved region of the leishmania *HSP60* gene. A single clone that hybridized strongly was selected and characterized. Sequence analysis revealed the presence of an open reading frame

of 1770 bp encoding a putative polypeptide of 589 amino acids with a predicted size of  $M_r$  64,790 and with the highest degree of amino acid sequence similarity (56%) to HSP60 from *T. cruzi*. Less extensive amino acid sequence similarity (48%) was observed between the leishmania HSP60 and the corresponding human protein. Notably, regions of significant sequence dissimilarity between the leishmania and human proteins were identified and these were concentrated principally within the carboxy-terminal regions of the proteins.

The entire coding region of the leishmania *HSP60* gene was amplified by PCR, subcloned into the pET-3a vector and expressed in *E. coli*. Purified recombinant protein was used to examine sera from patients with tegumentary leishmaniasis from South America for the presence of antibodies to HSP60. Unlike sera from healthy, uninfected controls, sera from patients reacted strongly with recombinant leishmania HSP60. In contrast, these same sera showed little or no reactivity with recombinant mycobacterial HSP65. To examine the protective potential of LHSP60, immunization studies were done in susceptible Balb/c mice. Recombinant leishmania HSP60 alone appeared to confer early (within the first 5 weeks) resistance against infection with *L. major* as determined by reduced lesion sizes in HSP60 immunized mice. In contrast, long-lasting protection did not develop in rLHSP60 immunized mice as lesion sizes and parasite numbers in these animals at 7 weeks of infection did not differ from those observed in control animals.

These findings provide evidence for the selective induction of leishmania HSP60 in infected macrophages and for the recognition of this protein in humans with leishmaniasis. Further studies of this protein should clarify its role in the host response to *Leishmania* and in disease pathogenesis.



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## LIST OF ABBREVIATIONS

APC	Antigen presenting cell
ATP	Riboadenosine 5'-triphosphate
ATPase	Riboadenosine 5'-triphosphatase
BMM	Bone marrow-derived macrophages
CFA	Complete Freund's adjuvant
cOVA	Chicken ovalbumin
CIDEIM	Centro Internacional de Investigaciones Medicas
CL	Cutaneous leishmaniasis
ClpA and ClpX	<i>E. coli</i> HSP100s involved in protein degradation/deaggregation
CMI	Cell mediated immunity
Con A	Concanavalin A
CPM	Counts per minute
CPR	Chicken progesterone receptor
CTLs	Cytotoxic T lymphocytes
CUP1	Metallothionein of <i>S. cerevisiae</i>
dATP	Deoxyriboadenosine 5'-triphosphate
dCTP	Deoxyribocytidine 5'-triphosphate
DEC	Dendritic epidermal cells
dGTP	Deoxyriboguanosine 5'-triphosphate
DHFR-TS	Dehydrofolate reductase-thymidylate synthase
DnaK	<i>E. coli</i> HSP70 homolog
DnaJ	<i>E. coli</i> HSP40, co-chaperone of DnaK
DTH	Delayed type hypersensitivity
dTTP	Deoxyribothymidine 5'-triphosphate
dUTP	Deoxyrobouracyl 5'-triphosphate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic-acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
$\sigma 70$	Factor transcribing most non-HS genes in <i>E. coli</i>
EtBr	Ethidium bromide
Ethanol	EtOH
FCS	Fetal calf serum
FtsH/HflB	Proteolytical component of <i>E. coli</i> involved in degradation of $\sigma 32$
GroEL	<i>E. coli</i> HSP60 homolog
GroES	<i>E. coli</i> HSP10, co-chaperonin of GroEL
Grp	Glucose regulated protein
HBSS	Hanks balanced salt solutions
HKLM	Heat killed <i>L. monocytogens</i>
HO	Heme oxygenase
Hr	Hour
HRP	Horseradish peroxidase
Hsc	Heat shock cognate protein

HSP	Heat shock proteins
HSE	Heat shock element
HSF	Heat shock factor
HtpG	<i>E. coli</i> HSP90 homolog
IB	Inclusion bodies
IC	Intracutaneously
IDDM	Insulin-dependent diabetes mellitus
IFA	Incomplete Freund's adjuvant
IEF	Isoelectric focusing
IFN- $\gamma$	Interferon gamma
IL-1	Interleukin-1
IL-12	Interleukin-12
IM	Inner membrane
IPTG	Isopropylthiogalactoside
Kb	Kilobase pair(s)
kDa	Kilodalton
IAP	Inhibitor of actin polymerization
LB	Luria-Bertani
Lb	<i>Leishmania braziliensis</i>
LCM	Liquid culture medium
LDA	Limiting dilution assay
Lma	<i>Leishmania mexicana amazonensis</i>
LMP	Low-melting-point agarose
L-NMMA	N <sup>G</sup> -monomethyl-L-arginine
LPG	Lipophosphoglycan
mAb	Monoclonal antibody
MCL	Mucocutaneous leishmasias
Mdj1p	Mitochondrial DnaJ homolog of <i>S. cerevisiae</i>
Mge1p/Yge1p	Mitochondrial grpE-homolog of <i>S. cerevisiae</i>
mt	Mitochondrial
Mts	Methallothioneins
MTT	3-(4,5-dimethylthiazol-2, 5-diphenyl tetrazolium bromide)
NaAc	Sodium acetate
NaAsO <sub>2</sub>	Sodium arsenite
(NANP) <sub>40</sub>	Repetitive sequence of <i>P. falciparum</i> circumsporozoite protein
NO	Nitric oxide
O <sup>•-</sup>	Superoxide anion
•OH	Hydroxyl radical
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
OD	Optical density
OMPs	Outer membrane proteins
OxyR	Hydrogen peroxidase-resistant mutant
PBMC	Peripheral blood mononuclear cells
PBP	Peptide binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor



pET-LHSP60	Plasmid containing the rLHSP60 gene
PfhsP 70	<i>P. falciparum</i> HSP70 antigen
pNPP	p-Nitrophenyl phosphate
P/S	Penicillin/streptomycin
RE	Restriction enzymes
rLHSP60	Recombinant leishmania HSP60
rMHSP65	Recombinant mycobacteril HSP65
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
rpoH	Gene encoding $\sigma_{32}$ factor
Scj1p	<i>S. cerevisiae</i> DnaJ homolog
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error medium
sHSP	Small heat shock proteins
$\sigma^E$	Factor that transcribes $\sigma_{32}$ at high temperatures
$\sigma_{32}$	Factor transcribing most heat shock genes in <i>E. coli</i>
SLA	Soluble leishmania antigen
ssa3	Stress seventy family A gene of <i>S. cerevisiae</i>
TBS	Tris buffered saline
TC1	<i>Trypanosoma cruzi</i> HSP70 antigen
TCP-1	T-complex polypeptide
TGF- $\beta$ 1	Transforming growth factor- $\beta$ 1
T-KMC	Tukey-Kramer Multiple Comparison Test
VL	Visceral leishmaniasis
YDJ1p	DnaJ homolog of <i>S. cerevisiae</i>

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## VII. INTRODUCTION

### A. STRESS PROTEINS

#### 1. Characteristics and properties

Heat shock proteins (HSPs) are highly conserved, ubiquitous molecules found in all organisms from prokaryotes to higher eukaryotes. They are expressed constitutively under basal conditions and may be induced further in response to increased temperatures (1). These responses, referred to as heat shock responses may also be induced by other stressful stimuli such as heavy metals, ionophores, amino acid analogs, inhibitors of energy metabolism and by pathophysiological states such as fever, inflammation, hypertrophy, reperfusion injury, viral and bacterial infection, neuronal injury and aging (2). Although most of these agents elicit expression of the classical heat shock proteins (HSP70, HSP60 and HSP90) and other stress proteins may only be induced in response to particular agents. Because multiple stimuli in addition to elevated temperatures induce increased expression of heat shock proteins, the heat shock response is also referred to as a stress response (1). Stress proteins have been classified into families based upon protein subunit size. These families include, HSP90, HSP70, HSP60, HSP100 and low molecular weight HSPs (15-30 kDa). An additional group is referred to as "less characterized" HSPs (3).

The HSP70 and HSP60 families, known as molecular chaperones, have been the focus of intense research in the last few years. Observations regarding their structure and function have led to new concepts of protein folding and assembly. In vivo assembly and folding of proteins may occur by assisted self-assembly with the help of molecular chaperones. Furthermore, molecular chaperones may work in tandem to facilitate folding. For example, HSP70 may bind to newly synthesized unfolded polypeptides thereby stabilizing them and then transfer them to HSP60 inside of which final folding takes place (3).

## 2. Regulation of stress proteins

### a. Regulation in eukaryotes

Molecular and biochemical studies of the heat shock response have been directed at understanding the factors that regulate induction of heat shock genes. It is now clear in higher eukaryotes that heat shock gene promoters have multiple copies of the invariant heat shock element (HSE) which is required for transcriptional induction. To initiate transcription, these sequences have to be bound by a regulatory protein known as the heat shock factor (HSF) (4).

Initial studies of the HSE were concerned with understanding its structure, organization and regulation. For example, analysis of mutations within the regulatory region of *Drosophila* HSP70 indicated that the HSE was localized between nucleotides -66 and -47 upstream from the HSP70 gene transcription start site. A consensus sequence of 14 bp within this region was later defined as CTGGAATTTCTAGA (5). Further analysis of 5' deletion mutants of the *Drosophila* HSP70 gene indicated that the 14 bp consensus sequence alone was not sufficient for full induction of the HSP70 gene (6). Examination of the wild-type HSP70 gene identified additional partial matches of the 14 bp consensus sequence further upstream. This suggested that more than one 14-bp HSE consensus sequence was required for induction of HSP70 in *Drosophila* to levels seen with wild-type alleles of the HSP70 gene (7). Both deletion mutant experiments and analysis of the regulatory regions of wild-type heat shock genes from *Drosophila* identified a simple repeating sequence nGAAn or its inverted repeat nTTCn (n=any nucleotide) which appeared to form the core recognition site of the HSE (6,7). Experiments were carried out to test the functionality of the nGAAn and nTTCn repeats. A synthetic regulatory construct containing two arrays of four contiguous inverted 5-bp repeats fused to the HSP70 gene of *Drosophila* was examined for activity in a transformation assay. It was found that the HSP70 gene was expressed at much higher levels than a HSP70 gene which contained only 2 native HSE (8,9). Additional studies in *Drosophila* HSP70 genes confirmed that each HSE contained a contiguous array of several nGAAn or its complement nTTCn (8). Furthermore, perfect or partial matches of the consensus sequence were found upstream in

other heat shock genes in *Drosophila* including, *HSP83*, *HSP27*, *hsp23* and *HSP22* (8). Similar consensus sequences were found in a variety of heat shock genes from different organisms including yeast and humans (10).

The heat shock factor (HSF) has also been investigated in respect to its structure, regulation and the mechanisms by which it binds to the HSE to activate gene transcription. The *HSF* gene is present as a single copy in both *S. cerevisiae* and in *Drosophila*. Three copies are present in tomato and chicken and at least 2 genes (HSF-1 and HSF-2) are found in mammals. HSF-1 is a monomeric phosphoprotein involved in activation of gene transcription in response to heat shock, heavy metals and amino acid analogs. Although HSF-1 is known to be phosphorylated in activated cells, the requirement for phosphorylation has not been fully established in terms of its ability to activate transcription (11). The function of HSF-2 is less clear, but evidence indicates that it may activate expression of *HSP* genes during normal processes such as cell differentiation (2). HSFs possess an overall amino acid sequence homology of less than 40%. They vary in length from 301 amino acids (aa) in tomato to 833 aa in *S. cerevisiae*. Three characteristics have been found to be conserved in HSFs: (i) an amino-terminal DNA binding domain (~ 100 aa), (ii) an adjacent trimerization domain containing an array of three hydrophobic heptad repeats (leucine zippers) that mediate oligomerization of the HSF during activation, and (iii) a leucine zipper located at the carboxy-terminus that appears to bind to the more NH<sub>2</sub>-terminal leucine zipper array to prevent trimerization of HSF under basal conditions (12).

Studies concerned with the mechanisms by which cells sense elevated temperatures and transduce this information to the nucleus to initiate gene transcription have suggested that HSP70 may negatively regulate this process. For example, studies in *Drosophila* revealed that underexpression of HSP70 results in extended synthesis and in overexpression of other stress proteins. Mutations in the *HSP70* gene of *S. cerevisiae* results in overexpression of the stress seventy family A gene (*ssa3*) placed under control of a HSE. This indicates that in the absence of HSP70 the HSF is free to induce the *ssa3* gene (2). It has also been found that inactive HSF present in cytoplasmic extracts obtained from non-heat shocked HeLa cells may

be activated to bind DNA by exposing the extract to either heat, non-ionic detergents or to low pH. Addition of HSP70 to this system blocks activation of the HSF and addition of ATP abrogates the inhibitory effect of HSP70 (2). Furthermore, introduction of either denatured or cross-linked proteins into *Xenopus* oocytes results in activation of the stress response. This suggests the possibility that abnormal proteins may sequester available HSPs in the cell thereby releasing and activating the HSF (13). The interpretation that HSP70 negatively regulates HSP expression is also consistent with results of experiments indicating that HSP70 is involved in thermotolerance. Thermotolerance correlates with high concentrations of HSP70. Conversely, loss of thermotolerance when cells are returned to normal temperatures is paralleled by decreased levels of HSP70 (2).

These and other observations have suggested a model for the regulation of *HSP* gene transcription in eukaryotes. In this model, accumulation of abnormally folded proteins within the cell triggers the stress response by activating the heat shock factor (HSF) which is found in a non-DNA binding form most likely stabilized by HSP70. During heat shock, the presence of denatured or unfolded proteins creates a large pool of protein substrates that bind to HSP70 resulting in release of HSF. Once "released," HSF becomes activated by unmasking its trimerization domain and exposing a hydrophobic surface which in turn forms hydrophobic coiled-coil interactions with two additional monomers. Trimerized HSF binds to the heat shock element (HSE) located in the promoter of heat shock genes resulting in their transcription. Thereafter, reaccumulation of HSP70 results in HSF binding and sequestration causing its deactivation and termination of transcription (2,12).

How the stress response and HSP synthesis becomes a rapid event is a matter of active investigation. It has been found for example in *Drosophila*, that RNA polymerase is already associated with HSP70 promoters prior to transcriptional activation. Upon heat shock, the transcription of the *HSP70* gene is initiated instantaneously as activated HSF binds to the HSEs in the promoter of the *HSP70* gene stimulating the rate of RNA polymerase elongation (14). Similarly, HSF from *S. cerevisiae* constitutively binds to DNA in the nucleus and undergoes

phosphorylation in response to heat and this results in rapid transcription of heat shock genes (12).

Of note, normal mRNA processing (i.e., splicing) has been found to be disrupted after heat shock treatment. However, mRNAs from heat shock genes are processed without interruption since they have particular structures that allow them to be transcribed selectively during stress. In fact, primary transcripts from HS genes (i) have sequences within 5'-untranslated regions conferring translational efficiency, (ii) have segments within 3'-untranslated ends which confer stability and although introns may be a common feature of HS genes (15), (iii) many HS coding sequences lack these structures (4).

#### **b. Regulation in prokaryotes**

Regulation of heat shock gene transcription in prokaryotes has been studied most extensively in *E. coli*. Seven heat shock promoters are known to be recognized by  $\sigma_{32}$ , the factor responsible for transcription of most heat shock genes.  $\sigma_{32}$  directs core RNA polymerase to recognize the promoters in HS genes (12,16,17). The sequences of these promoters have been used to derive a heat shock consensus sequence. This sequence (TCTCCCCTTGAA - 15 bp - CCCCAT - TA) is located at -35 to -10 bp, from the transcription start site (16). This is not a universal consensus sequence in prokaryotes since in *Bacillus subtilis* and in a group of gram-negative bacteria in which no  $\sigma_{32}$  has been found, a new heat-shock element termed CIRCE and a novel sigma factor have been found (18). Nevertheless,  $\sigma_{32}$  (32 kDa), encoded by the *rpoH* gene, is responsible for most HS gene transcription in *E. coli*. In fact, when *rpoH* is inactivated, either by insertional mutagenesis or by deletion of the entire coding sequence, the mutant *E. coli* strains are viable only at temperatures below 20<sup>0</sup> C. Similarly, when *rpoH*<sup>-</sup> strains are transfected with a multicopy plasmid carrying the GroE operon (encoding HSP60 and HSP10 homologs in *E. coli*) they grow at temperatures up to 40<sup>0</sup> C. Also when *rpoH*<sup>-</sup> strains are transfected with plasmids expressing both GroE and DnaK (HSP70 homolog of *E. coli*) genes, these strains grow at

temperatures up to 42<sup>0</sup> C. These observations suggest that the GroE and DnaK proteins are also required for *E. coli* to achieve growth at both normal and at high temperatures (16).

The gene encoding  $\sigma_{32}$  (*rpoH*) has at least four promoters (P1, P3, P4 and P5), the activities of which vary with temperature and growth conditions. Promoters P1 and P4 are recognized by  $E\sigma_{70}$ , a factor responsible for *rpoH* transcription under most growth conditions.  $E\sigma_{70}$  is also responsible for transcription of most non-HS genes in *E. coli* and may not recognize heat shock promoters (16). P5 is also recognized by  $E\sigma_{70}$  and is active during glucose deprivation or ethanol treatment. P3 is regulated by high temperatures particularly during lethal temperatures at 50<sup>0</sup> C when  $E\sigma_{70}$  appears to be inactive (16). Recent research indicates that P3 is regulated by another factor,  $\sigma^E/\sigma^{24}$ . The signals that induce  $\sigma^E/\sigma^{24}$  seem to arise in extracytoplasmic compartments such as the periplasmic space and outer membrane proteins (OMPs). Thus, accumulation of precursor forms of OMPs in the periplasmic space appears to induce  $\sigma^E/\sigma^{24}$  and underexpression represses it (19).  $\sigma_{32}$  is normally present at only a few copies per cell with rapid turnover ( $T_{1/2} = 1$  min.), most probably mediated by the heat shock protein FtsH/HflB, which seems to be a component of the proteolytic pathway in bacteria. Under heat shock conditions,  $\sigma_{32}$  concentration is increased both by improved translational efficiency and a decreased rate of degradation (12,16,17).

Recent research has begun to identify the mechanisms of how  $\sigma_{32}$  is regulated during heat shock. *E. coli* DnaK (HSP70) which constitutes up to 4% of total protein may play a key role. DnaK normally functions in an ATP-dependent manner with two other HSPs, GrpE and DnaJ (co-chaperones) (20). Studies using mutant strains of *E. coli* have demonstrated that DnaK, DnaJ, and GrpE may negatively regulate  $\sigma_{32}$  function. Thus, DnaK mutant strains overexpress HSPs at 30<sup>0</sup> C and fail to shut off the heat shock response at 42<sup>0</sup> C. Similar effects were observed when mutations were made in either DnaJ or GrpE. Furthermore, strains carrying mutations in either of these genes were also found to be defective in degrading  $\sigma_{32}$  and in reducing  $\sigma_{32}$  synthesis during termination of the heat shock response (16). The exact mechanism by which DnaK, DnaJ and GrpE negatively regulate  $\sigma_{32}$  is not completely understood, but it has been proposed that they may dissociate  $\sigma_{32}$  from core RNA polymerase



thereby making the  $\sigma_{32}$  factor susceptible to degradation. In this regard, both DnaK and DnaJ are capable of binding  $\sigma_{32}$  and sequestration of DnaK through binding to stress-denatured proteins, triggers activation of heat shock gene transcription apparently by releasing  $\sigma_{32}$  (16).

### **3. The HSP70 family**

#### **a. Structure and function**

Members of the eukaryotic HSP70 family are distributed in various cell compartments with some members being expressed in the basal state conditions and others induced by stress. Phylogenetic analysis of HSP70 proteins from diverse genera identified four distinct clusters which corresponded to their intracellular localization namely, cytosol, endoplasmic reticulum (ER), mitochondria and chloroplasts (21). Of note, mitochondrial HSP70s showed the highest degree of homology (60-77%) with the purple bacteria homolog, consistent with the hypothesis that these bacteria are the endosymbiotic progenitors of the mitochondria (21). Comparison of HSP70 sequences also indicated that the N-terminal two-thirds of these proteins are more conserved than the C-terminal one third. Analysis of bovine HSP70 indicates that the N-terminus contains an ATP binding domain between residues 380-390 the structure of which has been determined by X-ray crystallography (22). The C-terminal is less well conserved and contains the chaperone peptide binding sites. The crystal structure of a peptide complex with the substrate-binding unit of DnaK demonstrates that: (i) the structure consists of a  $\beta$ -sandwich subdomain followed by  $\alpha$ -helical segments, (ii) the  $\beta$ -sandwich subdomain (residues 393-502) comprises two sheets with four antiparallel  $\beta$  strands in each, (iii) the peptide is bound to the structure in an extended conformation through a channel defined by loops from the  $\beta$  sandwich, (iv) hydrogen bonds are formed between the peptide and the  $\beta$ -sandwich subdomain of DnaK, and (v) the  $\alpha$ -helical domain stabilizes the complex (23).

Recent in vitro studies have begun to identify mechanisms by which HSP70 is able to interact with target proteins. The C-terminus of BiP (HSP70 localized in the endoplasmic reticulum) has been found to bind peptides with an optimal size of 7 amino acids and

preferably with aliphatic side chains. Furthermore, it prefers peptides containing large hydrophobic amino acids particularly those containing Trp, Phe, and Leu in every second position in the heptapeptide motif (25). In general, the heptameric motif has been described as Hy(W/X)HyXHyXHyB, where Hy is an aromatic or hydrophobic residue (most frequently tryptophan, phenylalanine, or leucine, but also methionine and isoleucine), W is tryptophan, and X is any amino acid. Further analysis indicated that the bound peptides are in extended conformation with the aromatic/hydrophobic side chains lying on one side pointing to the binding cleft of the BiP molecule (25). These characteristics are consistent with the possibility that the structure of the peptide-binding domain of the HSP70 may be similar to that of MHC class I proteins. However, since the forces regulating the binding of peptide to the MHC binding cleft differ substantially from BiP-peptide interaction, the resemblance between the two systems remains controversial (25). Characteristics similar to peptide-BiP binding have also been observed in bovine hsc70 and it is believed that because of the high degree of homology in sequence and function between all members of the HSP70 family, similar peptide-binding site structures exist in all HSP70s (25). Furthermore, HSP70s bind unfolded and not native proteins. This may be explained by the fact that aliphatic residues are normally buried in folded proteins and are displayed on the surface only if the protein is denatured (26). This characteristic suggests that the presence of hydrophobic residues on the substrate protein may be a signal that allows recognition by HSP70 of unfolded versus folded polypeptides (27).

Biochemical analysis of HSP70 has identified diverse functions for this protein including binding of nascent polypeptides in ribosomes to avoid misfolding. This maintains translocation-competent conformation to allow import into the ER, mitochondria and movement across other membranes and modulates oligomeric assemblies (24). HSP70s maintains target polypeptides in a non-folded conformation in the presence of ADP (24). Binding ATP has been suggested to be sufficient to release HSP70 bound polypeptides in vitro. Thus, ADP enhances the affinity of HSP70 for peptide binding and the complex HSP70/ADP/peptide results in a more stable structure than does the HSP70/ATP/peptide

complex as determined by HPLC size-exclusion chromatography (24). In fact, HSP70 incubated with NCA staphylococcal nuclease at 37<sup>0</sup> C (unfolded) was found to form a stable complex. Addition of ATP resulted in dissociation of the complex with release of the substrate protein which was found to be in non-native conformation (24). Other findings indicate that mammalian cytosolic HSP70 binds to nascent polypeptides as they come off ribosomes (28). It has been suggested that attachment of the chaperone to newly synthesized polypeptides avoids incorrect interactions of the polypeptide with other molecules in an environment that is highly concentrated in proteins (in the range of 200-300 mg/ml) (3). Furthermore, in vitro crosslinking experiments demonstrate that DnaJ binds nascent ribosome-bound luciferase and chloramphenicol acetyltransferase. Subsequently DnaK and GrpE join the complex and this results in arrest of protein folding. In other studies, DnaK and GrpE were found to be required for translocation of DnaJ-bound protein into both microsomes and mitochondria (29). Thus, these data are consistent with the view that HSP70 binds unfolded polypeptides and maintains them in an unfolded conformation.

Another important function of HSP70 is to disrupt oligomeric protein complexes. In this regard both DnaJ and GrpE have been found to co-chaperone with DnaK. Together, the 3 HSPs form a functional unit known as "the DnaK chaperone machine". DnaK, DnaJ and GrpE are necessary for  $\lambda$  DNA replication in *E coli*. This process is believed to involve disruption by the "DnaK chaperone machine" of the protein complex formed at the origin of replication of  $\lambda$  phage. The complex consists of two  $\lambda$  proteins -O and P- and the host protein DnaB helicase. Once the complex is deaggregated, DnaG primase binds to the "opened" DNA resulting in synthesis of  $\lambda$  DNA (1). Similarly, in the plasmid P1-based model of DNA replication, it was shown that DnaK/DnaJ/GrpE convert inactive RepA dimers into monomers which bind avidly to oriP1 DNA and initiate plasmid DNA replication (30).

Based on the information summarized above a model for HSP70 action has been proposed. DnaJ interacts with polypeptide substrates as they emerge from the ribosome and targets DnaK to bind to the substrate. The ADP-bound state of DnaK favors binding to the polypeptide. DnaJ through its N-terminal conserved domain (the J region ), then specifically

accelerates the hydrolysis of DnaK-bound ATP by DnaK resulting in the formation of a stable peptide/DnaK/DnaJ complex. The weak ATPase activity of DnaK is further incremented by GrpE which also promotes release of ADP from DnaK. It has been estimated that together, DnaJ and GrpE account for an approximate 50-fold increase in the ATPase activity of DnaK. ATP then binds (not hydrolysis) to DnaK causing conformational changes in DnaK which promote release of bound polypeptide. The unfolded protein may now either bind again to DnaK/DnaJ, fold spontaneously or be transferred to the GroE chaperone system for final folding (3,12,31).

The roles of DnaJ and GrpE homologs as co-chaperones with HSP70 in eukaryotes has not been completely defined, but they may also modulate the activity of HSP70 as well. For example, in yeast a GrpE-related protein, Mgelp/Ygelp, is associated with the mitochondrial HSP70 and a complex of these proteins is required for translocation of proteins from the cytosol to the mitochondria (12). Similarly, the Mdj1p, a DnaJ-related protein that resides in the mitochondrial matrix is required for normal folding of imported proteins (32). Two additional proteins related to the DnaJ family in yeast have also been found in the ER either as an integral membrane protein (Sec63) or in the lumen (Scj1p). Both have been suggested to interact with Bip and to be part of the translocation/ folding pathway in this compartment (33).

## **b. Types of HSP70s**

### **b. 1. Glucose Regulated Proteins (Grps): BiP**

Glucose regulated proteins are found in the endoplasmic reticulum (ER). They are induced in response to glucose deprivation and multiple noxious stimuli including: hypoxia, calcium ionophore A23187, glucosamine, tunicamycin and reducing agents such as  $\beta$ -mercaptoethanol. Many of these agents affect glycosylation of proteins in the ER. Thus Grps are thought to be induced by the accumulation of nonglycosylated polypeptides in the ER (24). However, glucose starvation and hypoxia which do not alter glycosylation of proteins in the ER may induce Grps by affecting abnormal protein folding structure. Three Grps have been identified, Grp78 (BiP), Grp94 and the less studied 170 kDa Grp which was found to be

induced by glucose starvation (34). Some aspects of BiP are reviewed in this section while grp94 is reviewed in the section related to the HSP90 family.

The *BiP* gene has been cloned and sequenced from several species. Its amino terminus shares between 60-80% sequence homology with the amino terminus of the cytosolic hsc70 (24). BiP has an endoplasmic reticulum retention signal (K/H/D)DEL and studies indicate that BiP has a role in folding and assembly of newly synthesized proteins in the lumen of the ER. For example, BiP transiently interacts with immunoglobulin heavy and light chains. It has been suggested that it mediates proper assembly of the monomeric heavy and light chains into the mature form (35). Thus, it appears that BiP functions to stabilize and facilitate correct folding, oligomeric assembly or both of proteins that pass through the ER (35). Furthermore, BiP may also bind to proteins targeted for degradation. In the DR-A deficient cell line 9.22.3, BiP was found in a complex together with partially degraded DR- $\beta$  chain (36).

## **b.2. Cytosolic HSP70**

Two principal forms of HSP70's have been identified: (i) a highly inducible HSP72 and (ii) the constitutive HSP73 (also known as either constitutive HSP70, hsc70, or HSP70 cognate). In most cells HSP72 is synthesized at very high levels during stress but in primate cells it is expressed basally and is only slightly induced during stress (35). HSP72 resides in both the nucleus and the cytoplasm. In primate cells, its expression appears to be regulated during the cell cycle and is upregulated after transfection with oncogenes E1A or myc, during infection with adenovirus, polyomavirus, SV40 and herpes simplex virus type 2. It has also been found to be associated with the oncoprotein P53 (35). Constitutive HSP73 is also present in both the cytoplasm and the nucleus. It is an abundant protein in normal unstressed cells and is only slightly induced during stress (35). Both inducible HSP72 and constitutive HSP73 facilitate uncoating and release of clathrin triskelions from clathrin-coated vesicles in an ATP-dependent manner. Once the clathrin coat is removed the endocytic vesicle is able to fuse with other intracellular organelles such as the lysosome (35).

As discussed previously, cytosolic HSP70 is thought to stabilize unfolded polypeptides in the cytosol and maintain them in an unfolded conformation during translocation across membranes (37). The finding that proteins destined for the ER are associated with cytosolic HSP70 (38) supports this view. This may be a critical function of HSP70 since proteins being translocated across membranes are in an unfolded or extended conformation (39) and susceptible to aggregation (40). For example, the M13 precoat protein was observed to translocate into dog pancreas microsomes with the help of hsc70 and in the presence of ATP and limiting amounts of reticulocyte lysate. In this system, only cytosolic hsc70 increased the protease sensitivity of M13 precoat protein. This indicated that the polypeptide was in a translocation-competent state, a feature that was not observed when BSA was used instead of hsc70 (37,40). Furthermore, this function appears to be specific since neither DnaK nor BiP are able to substitute for hsc70 during translocation assays in yeast microsomes (41). Studies in yeast have suggested that in addition to HSP70, other HSPs that may include DnaJ and GrpE are also required for protein translocation into the ER (37).

### **b.3. Mitochondrial HSP70**

Protein import into mitochondria requires active involvement of the mitochondrial HSP70 (mt-HSP70) also known as Grp75 (75 kDa) which resides in the mitochondrial matrix. In this regard translocation of the peptide signal across the inner membrane (IM) and to the mitochondrial matrix is thought to depend on the energized IM, independent of mt-HSP70 (42). However, further translocation of the whole polypeptide into the mitochondrial matrix requires ATP and mt-HSP70 in a process that requires several cycles of binding and release of the protein being translocated from mt-HSP70 (35). The view that mt-HSP70 is involved in translocating proteins into mitochondria is supported by the findings that (i) yeast strains mutant for *mt-HSP70* gene are unable to import proteins into the mitochondria (42), (ii) mt-HSP70 is localized near the inner membrane of the mitochondria as determined by electron microscopic studies, and (iii) depletion of mitochondrial ATP impedes import of translocating proteins which instead accumulate on the outside of the mitochondrial surface (43).

#### 4. The HSP60 family

HSP60 is constitutively expressed and induced by stress in response to infection, inflammation, transformation or trauma (44-46). Members of the HSP60 family also play roles in DNA replication and mRNA turnover (45). Other evidence indicates that polypeptides newly imported into mitochondria are further folded to their native conformation by assisted assembly. This involves "help" from either specific protein complexes ("private" chaperones) or the specialized HSP60 (43). In eukaryotes HSP60 resides in mitochondria in animal cells and in chloroplasts in plants where it is involved in the folding of proteins in an ATP dependent manner. This enhances protein yield in conditions in which spontaneous folding does not occur (3).

In *E. Coli*, the *HSP60* gene forms an operon known as GroE which encodes two transcribed proteins, GroEL ( $M_r$  60,000) and GroES ( $M_r$  10,000). GroE constitutes about 1% of total cell protein at 30° C and 12% at 46° C (15). Both bacterial GroEL and the HSP60 from eukaryotes are organized as two seven-membered ring-like structures stacked one on top of the other in a "double-donut" structure of 60-kDa subunits. Each subunit containing an ATPase site. The ring-like structure encloses a central cavity of 6 nm diameter and 15 nm length. Non-native folding polypeptides bind to one end of the GroEL cylinder as determined by crystal structure (47). GroES is arranged in a single seven-membered ring. The crystal structure of GroES shows that the heptamer forms a dome with a cavity in it. The latter seems to be continuous with the polypeptide binding chamber of GroEL in the GroEL/GroES interaction model (48).

Recently, a chaperonin containing t-complex polypeptide 1 (TCP-1) also known as TF55, TRiC or TCC, was detected in the cytosol of eukaryotic cells (3). TCP-1 is a homolog of HSP60 and also has a homolog in archaebacteria (3). TCP-1 (57 kDa) resembles GroEL in that it is formed of two stacked rings containing a central cavity. However, unlike GroEL, each ring of TCP-1 contains eight or nine subunits instead of seven (3). Murine and human TCP-1 are cytosolic and exist as 800K-950 K hetero-oligomeric particles associated with

HSP70 (49). At least 9 different subunits of TCP-1 have been identified in mammalian testis and 7 of the genes have been cloned and found to share little sequence homology with each other (~ 30%). They do share, however, some highly conserved motifs indicating that they may have similar functions. Furthermore, each subunit was found to be highly conserved in different species from yeast to man (50). Of note, the high degree of sequence homology among the HSP60 protein family has been used recently to develop a phylogenetic analysis rooted at TCP-1. This identified unique sequence homologies the analysis of which support further the hypothesis that mitochondria originated by endosymbiotic capture of Gram-negative purple bacteria and that chloroplasts originated from *Cyanobacteria* (51). The functions of TCP-1 proteins are not fully elucidated, but they may assist in the assembly of multimeric proteins in the cytosol. Thus, TCP-1 has been found to chaperone folding of tubulin and actin in the cytosol in an ATP-dependent manner (12,52). TCP-1 was also observed to be involved in the formation of the mitotic spindle (12) and to be associated with a high molecular weight intermediate in the assembly of the hepatitis B virus capsid (53).

GroE proteins have been shown to be required for head assembly of bacteriophage lambda T4 and for tail assembly of T5 phage. In the case of  $\lambda$  phage, GroEL is involved in the oligomerization of the dodecameric lambda B protein which is located on the head, at the point of tail attachment (1). Overproduction of both GroEL and GroES in *E. coli* increases the solubility of the recombinant protein tyrosine kinase p50<sup>csk</sup> and this allows purification of high quantities of active enzyme (54). This and other in vivo observations suggest that GroE may assist protein folding into native structures.

Biochemical studies have begun to define the mechanisms by which GroEL binds polypeptides for folding. GroEL forms a binary complex with many unfolded proteins and, therefore, is regarded as a promiscuous protein that acts to assist protein folding in a general rather than a selective manner (3). It has been demonstrated in vitro that during binding to polypeptides, GroEL exposes hydrophobic surfaces that are hidden within itself and that this may stabilize the interaction of GroEL with the folding polypeptide (55). Consequently, GroEL preferentially interacts with side chains of hydrophobic amino acids such as Ile, Phe,



Val, Leu, and Trp and more weakly with polar or charged amino acids. On the other hand, GroES may reduce the specificity of GroEL for hydrophobic amino acids while increasing the specificity for hydrophilic ones (56).

Regarding the mechanisms of GroEL/GroES action, it has been observed that GroEL alone in the presence of ATP (without GroES) can successfully support spontaneous folding of a non-native polypeptide. However, this occurs only under specific solution conditions. Otherwise, in most cases, GroES has been found to be strictly required for successful chaperonin-assisted folding (57). It has also been observed that GroES-GroEL complexes are formed in the presence of ADP with GroES bound asymmetrically at one end of the GroEL cylinder forming a "bullet"-like structure with the folding protein substrate localized within the central cavity of GroEL (58). This structure has also been confirmed by electron microscopy (59) and by biochemical analysis where the stoichiometry involves one GroES heptamer per one GroEL tetradecamer coupled together to form a functional complex (3,58). Recent research, however, indicates that in the presence of ATP, GroEL and GroES associate symmetrically forming a "football"-like structure, with GroES at both ends of the GroEL cylinder. This has been regarded as an intermediate, transitory functional structure for chaperonin function (60). However, direct evidence for the function of this form is not available making the "bullet"-like structure the current accepted model of GroEL/GroES action (3).

The mechanisms by which GroEL, GroES and ATP interact to assist folding of proteins have also been studied. For example, it has been found that GroES and the folding protein counteract each other's effects on GroEL. Whereas GroES stabilizes GroEL in the ADP-bound state, binding of an unfolded polypeptide within the cavity of GroEL promotes GroES release. Furthermore, ATP hydrolysis induces release of the protein for subsequent folding and the partially folded protein binds again to GroEL (53). In this regard, ATP cycle experiments using GroEL/GroES and a substrate protein demonstrate that with a single round of ATP hydrolysis approximately 60% of the bound substrate leaves the complex in a non-

native form. This indicates that polypeptides can be released from GroEL in a non-native, unfolded state and that reassociation may improve folding (61).

Based upon available data, the 'Anfinsen cage' hypothesis has been proposed as a model of HSP60 assisted folding (3, 24). A partially folded protein (molten state) binds inside the HSP60 cavity. This is accomplished through the exposed hydrophobic motifs on the folding protein. The polypeptide becomes isolated in a dynamic cage in which the chain is now able to fold spontaneously. Addition of ATP and chaperonin 10 (GroES) causes ATP hydrolysis and results in release of the polypeptide which now folds spontaneously inside the cage. If the polypeptide is incompletely folded, it may enter the cycle again for a second round of folding. Thus, folding of the target protein occurs by multiple rounds of binding to and release from HSP60 which eventually produces a final folded structure. If on the contrary, the polypeptide is released because the groups interacting with the chaperonin inside the cavity are now buried, it leaves the cage. In this case, final steps for complete folding may occur outside the cage in the cytosol (3). However, cycling of a target protein is not a guarantee of proper folding. For example, unfolded  $\beta$ -actin or  $\alpha$ -tubulin were both shown to form complexes either with GroEL or HSP60 and both entered cycles of binding and release. However, neither achieved native protein structure (62).

Although the 'Anfinsen cage' model of chaperonin assisted-folding is generally accepted, an alternative model based on the 'iterative annealing' hypothesis, has been proposed. This argues that the function of the chaperonin complex is partly to unfold misfolded proteins (kinetically trapped intermediates) and that some of the energy of the ATP is channeled into the unfolded polypeptide. This forces it into a higher energy state from which it may once again attempt to fold correctly after being released into solution (48,63).

## **5. Sequential action of HSP70 and HSP60**

Available evidence indicates that proteins being imported into mitochondria are subjected to sequential interaction with mt-HSP70 (to keep the protein in an extended conformation) and HSP60 (to promote folding). As a result, newly imported proteins acquire native structures.

For example, functional inactivation of mt-HSP70 affects import of proteins into mitochondria while inactivation of HSP60 does not. This suggests that mt-HSP70, but not HSP60 is involved in protein import (43). On the other hand, in mitochondria depleted of HSP60, newly imported HSP60 is found associated with mt-HSP70. This suggests that because of the absence of preexisting HSP60, which is required for assembly of new proteins, newly imported HSP60 binds to mt-HSP70 since no chaperonin is available to "help" with final folding (64). Consistent with this view are the findings that in vitro, during importation of the  $\beta$ -subunit of the matrix processing peptidase ( $\beta$ -MPP) into yeast mitochondria, initially mt-HSP70 and then in successive steps HSP60 are co-immunoprecipitated with  $\beta$ -MPP (43). Furthermore, ATP hydrolysis promotes release of  $\beta$ -MPP from HSP60 and binding to mt-HSP70 (43). The sequential interaction of DnaK and GroEL seems to be directed by binding specificities. Thus, DnaK like various eukaryotic HSP70s exhibits high affinity for polypeptides that are in an unfolded conformation, lacking secondary structures. In contrast, GroEL stabilizes polypeptides in the process of folding in disordered tertiary structures (43). Also consistent with the view of the sequential actions of HSP70 and HSP60 are results indicating that in vitro purified chaperones DnaK, DnaJ and GroEL are able to assist native folding of chemically-denatured rhodanese (65).

## 6. HSP90 family

The HSP90 family comprises a group of molecules (87-92 kDa) found in the cytosol of both eukaryotes and prokaryotes. HSP90 of *E. coli* (HtpG) is essential for *E. coli* to grow at extremely high ( $46^{\circ}\text{C}$ ), but not lower temperatures ( $30^{\circ}\text{C}$ ). Mammalian HSP90 exists as a dimer with a rod-like structure that shares approximately 40% sequence homology with HtpG, is abundant under normal growth conditions and may be induced under stress (35,66). HSP90 has been found to bind cytosolic proteins and the interaction results in regulation of the biological activity of the target proteins. Both HSP90 and HSP56 have been found to interact with steroid hormone receptors maintaining them in an inactive form. When a steroid hormone binds to its receptor, HSP90 and HSP56 are released, the receptor oligomerizes,

translocates to the nucleus and binds DNA. Upon hormone withdrawal, the steroid receptor becomes deactivated binding again to HSP90 and HSP56 (1). In a reticulocyte lysate assay, purified chicken progesterone receptor (CPR) was found to bind HSP90 and HSP70 and three other proteins referred as p54, p50 and p23. Both p54 and p50 were found to be members of an immunophilin protein family which bind the immunosuppressant drug FK506 (67,68). HSP90 has also been found to associate with protein kinases and binding results in enzyme inactivation. For example, pp60<sup>src</sup>, a protein tyrosine kinase originally identified in Rous sarcoma virus, interacts with both HSP90 and a protein of 50 kDa. The enzyme appears to be inactive within the complex. Once the complex reaches the cellular membrane, pp60<sup>src</sup> is released and activated (1). Other protein kinases that bind to HSP90 include casein kinase, eIF2 $\alpha$  kinase, c-Raf-1 and calmodulin kinase (1).

A Grp-like protein known as endoplasmic reticulum protein or grp94 (94-108 kDa), related to the HSP90 family by sequence homology, has ER retention signals and localizes to the ER and the Golgi apparatus (1). In fact, grp94 like BiP has been found to interact with heavy and light chains of immunoglobulins. Unlike BiP, however, the interactions of grp94 with immunoglobulins is of longer duration, is unaffected by ATP and occurs with fully oxidized, more mature Ig molecules (69). Thus, grp94 seems to be a chaperone involved in late folding and assembly of proteins in both the ER and the Golgi apparatus (35,69).

## 7. Low molecular weight HSPs (sHSP)

Small heat shock proteins (sHSP) comprise a family of molecules of 15-30 (kDa) present in all eukaryotes, that share some features in common with the lens protein  $\alpha$ -crystallin (a sHSP residing in the lens). Their number is variable with at least 4 major proteins in *D. melanogaster*, 3 in mammals including the  $\alpha$ A- and  $\alpha$ B-crystallin, 1 in yeast and more than 20 in plants. Most sHSP possess a conserved domain (~80 amino acids long in mammals) known as the  $\alpha$ -crystallin domain located within the carboxy-terminal half of the protein (70).

Examination of sHSP and  $\alpha$ -crystallin molecules have defined two major domains. A less conserved domain located at the amino terminus (domain I) which appears to contain signal

peptides (first 15 amino acids) and a conserved phosphorylation sequence located at the C-terminus (domain II or  $\alpha$ -crystallin domain) which contains highly conserved regions shared by different species (70). Of interest, regions of homology in domain II have been found in surface antigens of several pathogens including the p40 egg antigen of *Schistosoma mansoni*, a 14-kDa antigen of *M. tuberculosis* and an 18-kDa antigen of *M. leprae* (70).

Human HSP27 and  $\alpha$ A and  $\alpha$ B crystallins are encoded by single genes. The regulatory mechanism responsible for accumulation of sHSP during stress is thought to operate at the transcriptional level. Although not completely defined, the mechanism of induction appears to involve a HSF that binds to a HSE located upstream in sHS genes, as is the case in other HSP families (70). In the basal state, expression of sHSP is highly variable in the various cell lines that have been examined. In some instances, expression is low making detection difficult. However, during heat shock or other types of stress sHSP are highly inducible (70).

The localization of sHSPs within cells varies between different organisms. In human cells HSP27 and the  $\alpha$ B-crystallin are localized in the cytoplasm, often close to the nucleus. In *Drosophila*, HSP23 is found in cytoplasmic granules while HSP27 is concentrated mainly in the nucleus. In *Neurospora crassa*, HSP22 and HSP30 are primarily in mitochondria. During heat shock or other forms of stress, most sHSP are localized near to or inside the nucleus and during cell recovery they gradually redistribute back into the cytoplasm (70).

All sHSP so far examined have been found to form oligomeric structures that sediment at 15S-20S in sucrose gradients and display molecular masses between 300 to 800 kDa. Murine HSP27 forms a spherical structure composed of 32 monomers arranged in a hexagon. During heat shock the size of the HSP27 complex increases leading to the formation of super-aggregated structures that redistribute inside the nucleus. The same phenomenon has been observed in  $\alpha$ -crystallins. The functional significance of the super-aggregation and redistribution of the sHSP to the nucleus is not clear, but it may be related to protection of nuclear structure (70,71). Recent research indicates that the sHSP may function as chaperones. Thus, sHSP including  $\alpha$ -crystallin suppress both aggregation and heat inactivation of proteins *in vitro* (72).

An intriguing feature of the sHSP is the ability of many of these proteins to become phosphorylated in response to various stimuli. Numerous agents are known to induce phosphorylation of mammalian HSP27 such as heat shock, arsenite, hydrogen peroxide, mitogens and diverse factors that induce differentiation including: thrombin, bradykinin, fibroblast growth factor (FGF), serum, platelet-derived growth factor (PDGF), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), phorbol ester, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and IL-1 $\alpha$  (70). Most of these agents induce rapid phosphorylation of HSP27 detectable within minutes without affecting any change in HSP27 levels. Heat shock, on the other hand, induces both phosphorylation followed by accumulation of HSP27 (73). Human HSP27 is phosphorylated at three sites identified as Ser-82, Ser-78, and Ser-15 which have been found not to be phosphorylated in a defined, ordered sequence (74). A heat-shock-induced HSP27 kinase has been purified from extracts of Chinese hamster ovary cells which appears to be formed of 2 polypeptides of 45 and 54 kDa. Furthermore, inactive pp45-54 HSP27 kinase purified from unstimulated cells was found to be activated by mitogen-activated protein (MAP) kinase purified from heat-shocked cells (70). Recent observations suggest that oxygen radicals may be involved in the signal transduction pathways that result in phosphorylation of HSP27. For example, both treatment of cells with scavengers of oxygen radicals and transfection of cells with the gene encoding glutathione peroxidase reduced or inhibited phosphorylation of HSP27 in response to either, H<sub>2</sub>O<sub>2</sub>, TNF- $\alpha$ , or serum (70,75).

Other findings suggest that mammalian HSP27 is a necessary component of a signaling pathway involved in polymerization of actin microfilaments. Evidence for this includes: (i) sequence analysis showing that chicken HSP27 is homologous to the inhibitor of actin polymerization (IAP), (ii) in Chinese hamster fibroblasts, HSP27 localizes in regions of highly motile cytoplasm such as the lamellipodia and ruffles which are active sites of actin polymerization, (iii) microinjection of antibodies against HSP27 in mouse cells blocks bombesin- and protein kinase C-induced sustained contractions (iv) overexpression of HSP27 in Chinese hamster cell lines increases concentrations of actin at the cell periphery and enhances the rate of pinocytosis, a process that is dependent on actin action, and (v) total F-

actin in hsp27-overexpressing cells increases ~ 2 fold in response to factors that induce phosphorylation of HSP27. Of interest, in control cells containing a plasmid that overexpresses nonphosphorylatable HSP27, little F-actin content is detected after stimulation with factors that induce phosphorylation of HSP27. Thus, nonphosphorylatable HSP27 appears to act as a dominant negative mutant (70).

It has been proposed that phosphorylation of HSP27 may cause conformational changes in the protein resulting in its dissociation from barbed ends of actin filaments. Free barbed ends would thus be able to add monomers for further actin polymerization (70). In the case of stress caused by heat, HSP27 phosphorylation may promote re-polymerization of actin filaments that are disrupted during stress (76).

## 8. Other Heat shock proteins

Other important HSPs include metallothioneins, a 32 kDa protein that may be involved in defense against oxidative stress and the chaperone HSP100, which may be involved in thermotolerance. Metallothioneins (MTs) are low molecular weight, cysteine rich heat shock proteins that bind metal ions. Their expression is induced in response to increased metal ion concentrations, various hormones, cytokines and other environmental stresses. Recently, it has been shown that a metallothionein from *S. cerevisiae* (CUP1) may be involved in defense against oxidative stress since it is induced by glucose starvation. Furthermore, while heat shock activates CUP1 transiently, glucose starvation activates CUP1 transcription for several hours and this has been interpreted to reflect differences in HSF action in response to different stimuli (77).

HSP100 comprises a highly conserved family of proteins found in most organisms including bacteria, yeast, plants, trypanosomes, and mammals, that possesses two ATP binding-domains. The two ATP-binding domains of HSP104 in *S. cerevisiae* carry out distinct functions. One is responsible for ATPase activity and the other is involved in controlling assembly of the protein into an oligomeric structure (78). HSP100 also appears to be important for survival under extreme conditions. In the absence of HSP104, survival of *S.*

*cerevisiae* is reduced as much as 1000 fold in comparison to wild-type. Conversely, when wild-type cells are pretreated at 37° C and then exposed to 50° C, the rate of cell survival increases by 10,000 fold (78). Recent research also indicates that HSP100 functions as a chaperone that mediates protein deaggregation since wild type HSP104, but not the mutant form was found to restore activity to luciferase aggregates that lacked activity as a result of exposure to high temperature (12). In prokaryotes, two members of the HSP100 family, ClpA and ClpX, appear to function as ATP-dependent regulators of the protease ClpP (12). ClpA forms an ATP-dependent hexameric ring which associates with ClpP. Addition of substrate peptides and ATP to this complex results in degradation of the peptides. Since ClpP synthesis increases about twofold upon heat shock, this suggests that the complex ClpA/ClpP is involved in degradation of proteins damaged during stress (78). Of note, ubiquitin (8 kDa), a HSP that is also induced after heat shock, is another well characterized example of a system that targets proteins for degradation.

Another stress protein of interest is a 32 kDa HSP observed to be induced in response to heavy metals (arsenite and cadmium), iodoacetamide, ultraviolet light, and oxygen radicals ( $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ ) (35). The deduced amino acid sequence of the 32 kDa protein from mouse 3T3 cells and from rat myoblasts revealed that this protein is highly related to heme oxygenase (see below) (35).

## 9. HSPs and the oxidative response

Reactive oxygen intermediates (ROI) such as superoxide anion ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and the hydroxyl radical ( $\cdot\text{OH}$ ) are unstable intermediates of  $\text{O}_2$  that result from the incomplete reduction of molecular oxygen. These radicals oxidize proteins, membrane fatty acids and damage DNA (79). To avoid damage to self structures, aerobic organisms must cope with these compounds. Synthesis of HSPs by cells on exposure to ROI appears to be one adaptative mechanism to cope with oxidative stress (80). In fact, in mammalian cells stress is associated with increased concentrations of ROI. Heat shock of murine peritoneal macrophages or the J774 cell line results in enhanced release of superoxide anion (81). Both



superoxide and hydroxyl radicals are increased in HeLa cells subjected to heat shock (82). Superoxide anion and  $\text{H}_2\text{O}_2$  may not be equally involved, however, in heat shock protein induction. For example, heat shock of HeLa cells at  $42\text{--}45^\circ\text{C}$  increases  $\text{O}_2^{\cdot-}$  production at least 6-8 fold as compared to cells incubated at  $37^\circ\text{C}$ , but increased superoxide production does not bring about HSP induction. On the other hand,  $\text{H}_2\text{O}_2$  which can be derived intracellularly from the dismutation of superoxide may be the critical active oxygen radical involved in heat shock protein induction in mammalian cells. Thus, in HeLa cells treated with MTT (to consume endogenous superoxide radicals thereby reducing production of endogenous  $\text{H}_2\text{O}_2$ ) and then heat shock, there is a remarkable reduction in HSP70 expression as compared to cells treated with heat shock alone. Since  $\text{O}_2^{\cdot-}$  generated during heat shock of HeLa cells did not induce HSPs, this suggests that endogenous  $\text{H}_2\text{O}_2$  produced at elevated temperatures contributes to induction of HSP70 in heat-treated cells (82). In agreement with this view are findings indicating that treatment of HeLa cells (82) or human macrophages (83) with  $\text{H}_2\text{O}_2$  increases production of a range of proteins including HSP70. Furthermore, incubation of mouse peritoneal macrophages (84), human skin fibroblasts (85) or peripheral blood monocytes (83) with  $\text{H}_2\text{O}_2$  results in increased synthesis of heme oxygenase (HO-1). Similarly, HSP23 was found to be induced in mouse macrophages in response to a variety of oxidative stresses including  $\text{H}_2\text{O}_2$ , glucose/glucose oxidase (which generates  $\text{H}_2\text{O}_2$ ), diethylmaleate, cadmium chloride, sodium arsenite, menadione, and zinc chloride (86). Furthermore, both HO-1 and HSP23 are induced in porcine aortic smooth muscle cells in response to oxidative stress brought about by either glucose glucose oxidase or diethylmaleate (87).

An additional HSPs are induced in macrophages during phagocytosis. For example, phagocytosis of sheep red blood cells by human macrophages induces the synthesis of proteins of 110, 90, 83, 70, and 32 kDa as determined by [ $^{35}\text{S}$ ]-methionine labeling (88). Partial peptide mapping of the 32-kDa protein identified it as heme oxygenase (89). Furthermore, exposure of human macrophages to either erythrocyte ghosts or to compound WR-1065 which can substitute for glutathione as a hydrogen donor prevents the induction of most of these

proteins. These results suggests that hemoglobin-derived iron and oxygen free radicals generated during phagocytosis of sheep red cells are involved in the induction of stress proteins (89).

Similarly, phagocytosis of inactivated *S. aureus* by human macrophages upregulates the expression of macrophage HSP70, HO-1 and superoxide dismutase (SOD). Since both HO-1 and SOD are considered scavengers of oxygen radicals, their induction suggests a protective role of these enzymes against oxidative stress during phagocytosis (80). In addition, human neutrophils exposed to either high temperatures or cadmium show increased synthesis of HSPs and also a selective, temperature-dependent inhibition of NADPH oxidase. This suggests that inhibition of NADPH oxidase may represent an intrinsic cellular mechanism to down-regulate production of superoxide (90).

A consistent feature of these studies is the finding that HO-1 is induced upon oxidative stress. In fact, HO-1 which is localized exclusively in the endoplasmic reticulum is induced in responses to a variety of stressful stimuli including, exogenous and endogenous chemicals, heavy metals as well as diverse factors such as X-irradiation, UV light, fever, starvation and bruising of the skin (91). Two forms of this enzyme have been described, HO-1 and HO-2. While HO-1 (32 kDa) is highly inducible HO-2 (36 kDa) is not. HO-1 has a heat shock regulatory element and likely numerous promoter elements to which inducers bind leading to gene transcription (91).

The inducibility of HO-1 in response to oxidative agents as well as its ubiquitous distribution has suggested that this enzyme is important in defense against oxidative stress. Consistent with this view are data indicating that rabbit endothelial cells transfected with human HO-1 gene exhibit increased resistance to the toxic effects of both recombinant hemoglobin and heme itself (92). HO-1 is involved in physiological degradation of heme to the bile pigment biliverdin, which after reduction is converted into bilirubin. Both biliverdin and bilirubin in particular have been found to be effective scavengers of oxygen radicals. Thus, HO-1 may protect cells against oxidative stress through the production of biliverdin and bilirubin (93).

## 10. Stress proteins and infection

HSPs may be particularly important for intracellular pathogens such as *Leishmania*, *Mycobacteria*, and *Toxoplasma* to survive within the host cell. Inside phagocytic cells pathogens have to cope with a variety of hostile elements including low pH, nutrient deprivation, high temperatures, proteases, nitric oxide (NO) and reactive oxygen intermediates (ROI). In this regard, evidence indicates that HSPs contribute directly to the capacity of microbial pathogens to survive within cells. *E coli* cells infected with bacteriophage lambda express the HSP GroEL which is essential for maturation of the virion (94). Studies also indicate that the homolog of GroEL, mammalian HSP60, as well as mammalian HSP70 and HSP90 are induced in virally infected animal cells (94).

Treatment of *Salmonella typhimurium* with H<sub>2</sub>O<sub>2</sub> results in the induction of at least 30 proteins. Nine of these proteins are also overexpressed in the basal state in a hydrogen peroxidase-resistant mutant strain (oxyR). Mutant strains of *S. typhimurium* in which the oxyR-regulated genes are constitutively expressed are resistant to killing by H<sub>2</sub>O<sub>2</sub>. Conversely, strains unable to induce the oxyR-regulon have enhanced H<sub>2</sub>O<sub>2</sub> sensitivity and unlike the parent strain, are unable to survive inside macrophages (95). Of interest, 5 of the 30 proteins induced by H<sub>2</sub>O<sub>2</sub> (including DnaK) are also induced by heat shock and one of these 5 proteins is regulated by the oxyR regulon (79). Consistent with these results are findings indicating that *S. typhimurium* overexpresses both GroEL and the HSP70 DnaK during infection of macrophages and the presence of these HSPs is essential for survival of the pathogen within the infected cell (94,96).

At least 16 *M. tuberculosis* proteins are induced in human THP-1 cells infected with a virulent strain of the bacterium. Some of these proteins are also induced by growing the bacteria extracellularly in culture medium in the presence of heat shock, H<sub>2</sub>O<sub>2</sub> or low pH (97).

HSPs may play an essential role in the survival of parasites that invade homeothermic (37°C) hosts. In this regard, *Leishmania*, *Trypanosoma*, *Plasmodium*, *Schistosoma*, nematodes and

the yeasts *Histoplasma* and *Candida* have all been found to express constitutive or induced forms of HSPs at high concentrations (98). Four genes corresponding to HSP70 have been cloned from schistosomes. They are expressed constitutively in larvae and in adult organisms. At least one of these HSP70s is inducible in *S. mansoni*. In fact, schistosomula of *S. mansoni* are unable to mount a heat shock response during the first 3 hr of this differentiated stage. However, fully differentiated schistosomula can induce the HSP70 gene. Furthermore, an *HSP70* gene has been found to be constitutive in miracidia and in adult worms but not in cercaria. In cercaria, however, the gene is induced at 42<sup>o</sup> C (98,99).

Optimal expression of HSPs appears to vary between strains with differing degree of virulence in the yeast *Candida albicans*. Thus, nonvirulent strains transcribe both *HSP70* and *HSP82* at high levels at 34<sup>o</sup> C. Conversely, virulent organisms expressed maximal transcription of these *HSPs* genes at 37<sup>o</sup> C. Similar results are also found at the protein level (100).

*Theileria anulata*, a tick-borne parasite, expresses high levels of HSP70 at 28<sup>o</sup> C and the gene is induced further by HS at 37<sup>o</sup> C (98). At least 10 proteins with dominant bands between 60 to 83 kDa show increased expression in *T. cruzi* epimastigotes treated at 37 to 41<sup>o</sup> C. Furthermore, treatment of epimastigotes of *T. cruzi* with HS at 37<sup>o</sup> C induces the *HSP70* gene by 4-fold. A similar increase is observed when parasites reach stationary phase in culture at 28<sup>o</sup> C (101). HSP70 and HSP83 mRNAs are augmented up to 100 fold in trypomastigotes of *T. brucei* at 37<sup>o</sup> C as compared with forms found in the insect vector (102). At least 6 *HSP70* genes have been found in *T. brucei* in a locus of 23 Kb all of which are transcribed as a long polycistronic molecule (98).

Several *HSP70* genes have been cloned and sequenced in *Plasmodium falciparum*. The proteins encoded by these genes are abundant and constitutively expressed at all blood stages of the parasite. Interestingly, one member of the HSP70 family, a 75 kDa protein, is expressed on the surface of merozoites as determined by immunoelectron microscopy (103,104).

Also of note, the dimorphic pathogen *Histoplasma capsulatum* has been found to maintain mRNA processing at high temperatures even when phase transition is induced by culture at 42° C. Since HSPs are abundant in these organisms at normal temperatures and during phase transition, it has been suggested that HSPs, particularly HSP70, may promote normal mRNA processing and normal cell function at 37° C by protecting the spliceosome (105).

### **11. Stress proteins and the host immune response during infection**

HSPs are among the most immunogenic antigens found. It has been suggested that the immunogenicity of these proteins is a direct consequence of their abundance which by virtue of mass action leads to processing and presentation by antigen presenting cells (1,106). It has also been suggested that the immunogenicity of at least some HSPs may be related to their functional association with the MHC processing machinery (107). In support of this view, it has been observed that (i) two genes of the HSP70 family are located within the MHC gene complex, (ii) a peptide binding protein (PBP) which has been proposed to be involved in peptide loading of MHC class II, is a member of the HSP70 family, and (iii) HSP70 and HSP90 chaperone immunogenic peptides derived from tumor cells (107).

In any case, HSPs appear to be highly immunogenic in their own right. Antibodies and T cells that recognize HSPs have been identified in a variety of infections and also in apparently healthy individuals (44,46). The latter finding has led to the suggestion that HSPs may play an important role in immune surveillance. Thus, anti-HSP immune responses appear to be regularly induced as a result of frequent contact with low virulence microorganisms. Repeated contact with low virulence pathogens impels the immune system to focus on regions of HSPs conserved in the microbial world. This may provide a mechanism for rapid and specific responses to eventual encounters with more highly virulent microbes (44,46,107).

In respect to HSPs as immunogens during infection, evidence indicates that HSP70 and in some cases the HSP90 are major targets of the immune response in parasitic infections. In contrast, GroEL appears to be the major target of the mammalian immune response to bacterial infections (see below) (59). For example, Pfhs 70-1 and Pfhs 70-2 are two abundant

antigens of *P. falciparum* HSP70 that are expressed at all stages of the parasite life cycle. Although they share 64% amino acid identity, antibodies raised against either of them do not show crossreactivity indicating that the common sequences are non immunogenic (108). The Pfhsp 70-1 antigen is expressed on the surface of infected hepatocytes where it is the target of antibody-dependent cell-mediated cytotoxicity (21). Recently, a major HSP70-related immunogen Pf72/hsp70-1, which is present in blood stages of *P. falciparum* has been found to protect *Saimiri* monkeys against infection. Fifty-two percent of individuals living in an endemic zone in West Africa have antibodies to this antigen. Furthermore, T cells specific for epitopes within the C-terminus of this protein are found in individuals continuously exposed to the parasite. The same epitopes are not recognized by T cells of non exposed Europeans. However, since some of these T cell epitopes are also present in the homologous human HSP70, the use of this antigen in vaccine development against malaria remains controversial (109). An antigen of *P. falciparum* which shares 55% amino acid identity with Pfhsp 70-1 and 72% identity with rat grp78 is also recognized by sera from infected patients (110).

In *T. cruzi*, antigen TC1 that belongs to the HSP70 family has been cloned from a  $\lambda$ gt11 expression library. Antibodies against this antigen do not cross react with human HSP70 despite 73% amino acid homology between the proteins (111).

A HSP70 molecule from *S. mansoni* is recognized by sera from *S. mansoni*-infected individuals. The same molecule is not recognized by sera from patients infected with *S. japonicum* indicating that the two groups of sera recognize different epitopes within the two HSP70's (112,113). HSP70 is also a major B cell antigen in patients infected with either *Brugia malayi* or *Onchocerca volvulus*. The immunogenic domain in both cases has been localized to the carboxy-terminus (114).

HSPs have been found to be major targets of the immune response in bacterial infections. Mice immunized with either *M. tuberculosis* or *M. leprae* produce antibody responses to a limited set of proteins. Amongst these are represented at least 4 stress protein groups: HSP70, HSP60, HSP18 and HSP12 (106). HSP70 was initially identified by a mAb raised against an extract of *M. leprae*. HSP70 was then found to be recognized by both T cells and antibodies

in patients with leprosy (115). Similarly, HSP70 from *M. tuberculosis* was also identified with a mAb raised against *M. tuberculosis*. This HSP70 is also recognized by both antibodies and T cells from patients with tuberculosis (116). Mice infected with *M. tuberculosis* develop a strong antibody response to mycobacterial HSP70 and little or no response to murine HSP70. However, immunization of mice with the mycobacterial HSP70 induces antibodies that cross-react with self HSP70. Furthermore, CD8 T-cell clones isolated from patients with tuberculosis proliferate in response to HSP70 from mycobacteria, *E. coli*, and human (117).

Some low molecular weight heat shock proteins (sHSP) have also been found to be immunogenic. For example, in patients with tuberculoid leprosy, approximately one third of *M. leprae* reactive T cells that respond to the whole organism also respond to GroES (HSP10) (118). In another study, peripheral blood mononuclear cells from 18 of 19 healthy lepromin-positive contacts of leprosy patients were found to proliferate or produce Th-1 cytokines in response to the 10-kDa *M. leprae* GroES, suggesting that this antigen may be involved in protection (119).

## 12. The carrier effect of HSPs in vaccine constructs

Recent research indicates that some HSPs may have intrinsic adjuvant properties. When conjugated to poorly immunogenic peptides or oligosaccharides they enhance the immune response to these relatively weak antigens. For example, immunization of mice with the polypeptide (NANP)<sub>40</sub> [a repetitive sequence of the *P. falciparum* circumsporozoite (CS) protein] conjugated to mycobacterial HSP60 or HSP70 results in a strong anti-peptide IgG antibody response. This is similar to the response observed when PPD is used as a carrier in spite of the fact that no conventional adjuvant is used in the case of the HSP-conjugated peptides (120). Further experiments indicated that in order to obtain maximal anti-peptide antibody production, priming with BCG prior to immunization is required in cases when HSP60 is used as a carrier. However, priming with BCG is not required when using HSP70 (120).

This same approach of immunization has been applied to other antigens and to other animal species including non-human primates with similar results (121). The mechanism by which HSPs mediate strong helper effects in vivo in the absence of adjuvant is not clear. One explanation may be that the adjuvant effect of the HSPs is due to HSP-specific T cells which are naturally present at the time of immunization. These HSP-specific T cells presumably exist as a result of previous encounters of the organism with microbial HSPs (121,122). In support of this view are findings that vaccination of children with a trivalent vaccine against tetanus, diphtheria, and pertussis induces high titers of anti-HSP antibodies. This response appears to be related to the presence of whole-cell pertussis components of the vaccine (containing HSPs), since it is not observed in infants receiving an acellular vaccine containing tetanus and diphtheria toxoids combined with a genetically inactivated pertussis toxin known as PT-9K/129G) (122). More recently, an approach to immunization using the carrier properties of the HSP60 has been proposed. It takes advantage of the presence of natural T cell reactivity to HSP60 in healthy individuals. Thus, a series of homologous T cell immunodominant peptides are synthesized from different HSP60s, conjugated to the poorly immunogenic capsular polysaccharide antigen of *Salmonella typhi* (Viag) and tested for their immunogenicity in Balb/c mice in the absence of adjuvant. In this model, HSP60-Viag induces high titers of anti-polysaccharide antibodies of the IgG1 type. The HSP60-peptide carrier effect is T cell dependent and was not associated with autoimmune responses (123).

### **13. The HSPs as chaperones of antigenic peptides**

Recently, a novel method for immunization against cancer using the peptide-binding property of HSPs has been explored. Mice immunized with HSP70 purified from Balb/c Meth A sarcoma cells were found to be protected against an otherwise lethal challenge with tumor cells. Protection is specific since it is not obtained when mice are immunized with either (i) HSP70 from normal tissue, or (ii) HSP70 treated with ATP which removes HSP70-bound immunogenic peptides. Thus, protection appears to require a combination of HSP70 and co-purifying bound peptides. Further analysis indicates that bound peptides are in the range of



1000 to 5000 daltons (124). Similar effects are obtained using HSP90 or gp96 purified from cancer cells (125). Furthermore, the murine macrophage cell line J774 transfected with mycobacterial HSP65 is no longer able to produce tumors in syngeneic mice (126). Although the mechanism involved here is not clear, it has been suggested that HSP-chaperoned peptides are more efficiently processed endogenously by the macrophage and presented in the context of MHC class I molecules. In support of this hypothesis, are findings showing that murine macrophages pulsed with the nucleocapsid protein of vesicular stomatitis virus (VSV) in combination with the HSP, gp96, are recognized and lysed by VSV-specific cytotoxic T lymphocytes (CTLs) more efficiently than if the gp96 is not included (127). Thus, it has been suggested that HSPs are biased towards binding or chaperoning antigenic peptides generated through MHC processing or that HSPs enter preferentially into the MHC class I processing pathway (128).

#### 14. Summary

HSPs are highly conserved among widely divergent organisms. Both HSP60 and HSP70 have about 50% of their sequences conserved between human and *E. coli* homologs. They are found distributed in most compartments of the cell. For example, members of the HSP70 family have been found in mitochondria, nucleoli, endoplasmic reticulum and cytoplasm. HSP90 has been localized in the cytosol while HSP60 is found mainly in mitochondria in animal cells and in chloroplasts in plants.

HSP70 (DnaK in *E. coli*) is co-chaperoned by the HSPs DnaJ and GrpE (known as the DnaK chaperone machine) the main function of which is to bind unfolded polypeptides. This maintains them in an extended conformation either to facilitate further folding or to assist with translocation across membranes. On the other hand, HSP60 (GroEL in *E. coli*) binds unfolded polypeptides and with the assistance of HSP10 (GroES in *E. coli*) helps them acquire native quaternary structures. Similarly, HSP90 (HtpG in *E. coli*) associates with certain protein kinases such as pp60<sup>src</sup>, and transcription factors, such as glucocorticoid receptors and inhibits their activity until proper conditions for their function are met. HSP90 has also been found to

associate with a number of cohort proteins including HSP56 and HSP70. HSP100 on the other hand, appears to be essential for survival under extreme conditions (46<sup>o</sup> C) and for acquisition of thermotolerance. Furthermore, HSP100 functions in an ATP-dependent manner in the regulation of the heat-induced protease ClpP in *E coli*.

The small heat shock proteins (sHSP) (15-30 kDa) are present in eukaryotic cells and share common features with the  $\alpha$ -crystallins (sHSP residing in the lens). They have been found to form oligomeric structures that increase in size during heat shock. Some sHSP become phosphorylated under stress or during stimulation with mitogens and phosphorylation of HSP27 in mammalian cells correlates with actin polymerization and mitotic spindle formation.

Reactive oxygen intermediates have the potential to cause serious damage to proteins, membrane fatty acids and DNA. There is substantial evidence to suggest that induction of HSPs by ROI may provide protection against oxidative injury. For example, (i) macrophages increase synthesis of ROI upon stress, (ii) intracellular production of H<sub>2</sub>O<sub>2</sub> induces HSP70 in HeLa cells, (iii) treatment of cells with H<sub>2</sub>O<sub>2</sub> induces HSP expression including HSP70, HSP23, and heme oxygenase, (iii) phagocytosis induces synthesis of a wide range of stress proteins such as NADPH oxidase and heme oxygenase, both known to be ROI scavengers, and (iv) rabbit endothelial cells transfected with the heme oxygenase gene are more resistant to the toxic effects of hemoglobin and heme compounds than are nontransfected cells.

On the other hand, HSPs may be important for intracellular pathogens to survive the hostile environment of the host cell which is characterized by high temperature, low pH, nutrient deprivation, the presence of proteases, nitric oxide (NO) and ROI. In this regard, evidence indicates that invading pathogens selectively express abundant amounts of HSPs. *S. typhimurium* overexpresses a variety of HSPs including DnaK and GroEL during infection of macrophages and the presence of these HSPs appears to be essential for survival of the pathogen within the infected cell. Similarly, *Leishmania*, *Trypanosoma*, *Plasmodium* and *Schistosoma* have been found to express constitutively diverse groups of HSPs that include HSP70 and HSP83. Furthermore, members of the HSP70 family are abundantly expressed at

all stages of the life cycles of these parasites and some forms are induced upon incubation at 37° C.

HSPs are amongst the most immunogenic proteins known. Antibodies and T cells against HSP83, HSP70, HSP60, and low molecular weight HSPs have been found in many infectious diseases. While most studies identify HSP70 as an immunogenic protein in parasitic infections, HSP60 is mainly involved in recognition during infections with bacteria (see next section). In *P. falciparum*, Pfhsp 70-1, Pfhsp 70-2, and Pf72/hsp70-1 are well characterized B cell antigens. Antibodies against HSP70 have also been identified in schistosomiasis, filariasis, African trypanosomiasis, and Chagas' disease. T cells recognizing HSP70 have been demonstrated as well in infections with mycobacteria both in humans and in animal models. T cells against GroES are found in patients with tuberculoid leprosy.

Peptide binding by some HSPs has been used recently to prepare highly immunogenic vaccine constructs. In this approach, the HSP is coupled to poorly immunogenic peptides and the complexes are used for immunization of animals in the absence of conventional adjuvants. This results in strong B cell responses against HSP-coupled peptides. Furthermore, HSPs appear to bind immunogenic peptides in vivo. For example, mice immunized with HSP70 derived from tumor cells are protected against challenge with lethal doses of the same tumor cells. Protection appears to be conferred not by the HSP itself, but by the bound peptide while the chaperone provides amplification.

## **B. IMMUNE RESPONSE TO HSP60**

### **1. HSP60 as a B and T cell antigen**

HSP60 has been shown to be highly immunogenic for both B cells and T cells (44,106). HSP60 has been referred to as a 'common antigen' because immune recognition of this molecule occurs in a variety of human infections that involve either bacteria, protozoa or metazoa. Antibodies specific for HSP60 have been described in malaria (129,130), visceral (131) and cutaneous leishmaniases, Chagas' disease, schistosomiasis (112,132), leprosy, tuberculosis, Q fever, syphilis, Legionnaire's disease (133), Lyme disease, ocular trachoma,

brucellosis (in cattle) (134), aspergillosis (135), borreliosis, (136) and during infection with *Chlamydia* and *Neisseria* (137).

B cell epitope mapping using synthetic peptides and monoclonal antibodies to recombinant HSP60s from *M. leprae*, *M. tuberculosis* and GroEL have demonstrated that these antibodies: (i) map to predominantly linear determinants, (ii) recognize epitopes distributed throughout the sequence of the protein, and (iii) are in some cases species-specific and in other instances are cross-reactive (108).

T cells with specificity for mycobacterial HSP65 have been identified both in mice and in humans. For example, in mice immunized with *M. tuberculosis*, 10-20% of T cells respond both to whole *M. tuberculosis* organisms and mycobacterial HSP65 (138). A CD4<sup>+</sup>, class II restricted T cell clone isolated from mice immunized with *M. tuberculosis* was found to respond to mycobacterial HSP65 with the production of IFN- $\gamma$  and IL-2 (106). In both tuberculosis and leprosy patients, mycobacterial HSP65 specific T cells have been repeatedly identified. In the range of twelve determinants recognized by human T cells have been mapped in mycobacterial HSP65 (106).

HSP65-specific T cells obtained from PPD<sup>+</sup> human donors that respond in vitro to the HSP65 lyse autologous monocytes pulsed with HSP65. These cells are CD4<sup>+</sup>, MHC class II restricted and the epitopes recognized appear to be within the carboxy-terminus of the molecule (139). Furthermore, T cells with reactivity to HSP65 have also been identified in individuals without any clinical signs of disease (140). It has been suggested that the presence of these HSP60-reactive T cells in healthy individuals may reflect prior, subclinical infections with organisms expressing HSP60 and that they may contribute to immune surveillance and host defense (140,141). In support of this view, are findings indicating that murine macrophages "stressed" either by cytomegalovirus infection or by IFN- $\gamma$  stimulation express HSP65 and are recognized and lysed by mycobacterial HSP65-specific CD8<sup>+</sup> cytotoxic T cells (142).

## 2. HSP60 and gamma delta ( $\gamma/\delta$ ) T cells

The  $\gamma/\delta$  TCR is the first CD3-associated receptor to appear during thymocyte development and defines a separate T cell lineage distinct from TCR  $\alpha/\beta$ -bearing cells.  $\gamma/\delta$  cells constitute 1-10% of peripheral T cells in both man and mouse and they account for 30% in the chicken.  $\gamma/\delta$  T cells are predominantly  $CD4^-CD8^-$  with small proportion being  $CD4^-CD8^+$  and still fewer being  $CD4^+CD8^-$  (143). These cells preferentially colonize nonlymphoid tissues, particularly epithelia in the mouse and often segregate at a given site into tissue specific subsets with characteristic clonal composition. The best example of this are dendritic epidermal cells (DEC) which express striking homogeneity among their  $\gamma/\delta$  receptors.  $\gamma/\delta$  cell populations with distinct clonal compositions have also been found in the gastrointestinal tract, lung, female reproductive tract and lactating mammary glands (144).

The strategic localization of  $\gamma/\delta$  T cells in murine epithelia and their limited diversity has lead to the suggestion that these cells may constitute a first line of host defense and represent an ancient surveillance system. According to this model, these cells recognize monomorphic molecules, the prime candidates being HSPs (144). In fact,  $\gamma/\delta$  T cells have been found to respond to HSP60. For example,  $\gamma/\delta$  T cell hybridomas from newborn mice respond to mycobacterial HSP60 and the majority of these cells have polyclonal receptors of the  $V\gamma 1^+$  type (143). Human peripheral blood  $\gamma/\delta$  T cells recognize and lyse Daudi Burkitt's lymphoma cells and lysis is inhibited either by a mAb specific for the  $\gamma/\delta$  TCR or with antibodies against human HSP60. A  $\gamma/\delta$  T cell line derived from peripheral blood mononuclear cells of a PPD reactive, BCG immune individual also showed a specific proliferative response to recombinant mycobacterial HSP65 (145). Similarly, a  $\gamma/\delta$  T cell clone from a healthy donor was shown to recognize both the 65 kD mycobacterial HSP as well as a recombinant human homolog of this HSP (146). A  $\gamma/\delta$  T cell clone isolated from the synovial fluid of a patient with rheumatoid arthritis was found to respond to mycobacterial HSP60 (143). In addition a synthetic peptide (amino acids 180 to 196) of *M. leprae* HSP65 was found to stimulate several PPD-reactive  $\gamma\delta$  T cell hybridomas. This peptide overlaps with an epitope recognized by arthritogenic  $\alpha/\beta$  T cell clones (147).

Of interest, human monocytes infected with viable *M. tuberculosis* are effective inducers of  $\gamma/\delta$  T cell expansion. In contrast, when heat killed *M. tuberculosis* is used as a source of antigen mostly  $\alpha/\beta$  T cells are elicited (148). In mice, treatment of lymph node cells at 42<sup>0</sup> C for 30 min followed by incubation at 37<sup>0</sup>C results in selective proliferation of  $\gamma/\delta$  T cells. This effect is augmented by preexposure of mice to mycobacterial antigens (149). These data suggest that both live bacteria and heat shock induce the expression of HSP on the surface of accessory cells where recognition by specific  $\gamma/\delta$  T cells may occur.

### 3. HSP60 cell surface expression

Evidence indicates that epitopes of HSP60 are expressed on the surface of target cells where they are recognized by either HSP60 specific T cells or antibodies. For example, Schwann cells or macrophages stressed either by virus infection or by interferon- $\gamma$  are recognized and lysed by mycobacterial HSP60-specific T cell clones (142,150). A monoclonal antibody specific for an epitope of human rHSP60 has been shown to recognize an epitope on mouse bone marrow derived macrophages (BMM). The intensity of recognition is increased when BMM are stressed with IFN $\gamma$  (151). In another study it was found that GroEL of *S. typhimurium* is involved in binding of the bacterium to intestinal mucus in a guinea pig model suggesting the possibility that the protein is expressed on the bacterial cell surface (152). Recently, it has been shown that mAb II-13 raised against human HSP60 recognizes an epitope on the surface of Daudi Burkitt's Lymphoma cells and immunoprecipitates HSP60 from the surface of these cells (153). Thus, epitopes of HSP60 are frequently expressed on the cell surface where they may be targets of the immune response.

### 4. HSP60 and autoimmunity

In humans, evidence suggests that HSP60 is involved in various forms of autoimmune disorders. For example, T cells specific for mycobacterial HSP60 have been isolated from the synovial fluid of patients with various forms of arthritis (106). A T cell clone isolated from the synovial fluid of a patient with reactive arthritis was shown to respond to both

mycobacterial and human HSP60 (154). Epitope mapping using both mycobacterial and human HSP60 showed that T cells from patients with Behcet's Disease specifically recognize mycobacterial HSP60 peptides as well as homologous, human HSP60 peptides (155). In another study, human T cell lines and clones recognizing 5 synthetic T cell epitopes of *M. leprae* HSP65 were tested for specificity. Three clones were found to be specific for mycobacterial HSP65 and two were found to recognize both the mycobacterial protein and the human homolog (156). In reactive arthritis induced by yersinia infection, T cell responses were found to be directed towards epitopes shared by both mycobacterial HSP65 and human HSP60 (156). Furthermore, T cells from synovial fluid and peripheral blood of patients with juvenile chronic arthritis are significantly activated in vitro with human HSP60 (157). These findings suggest that HSP60 specific T cells may be involved in autoimmune reactivity when they recognize conserved regions common to both host and pathogen HSP60.

There is also evidence to indicate that HSP60 may be involved in autoreactivity in experimental autoimmune diseases. T cells involved in insulin-dependent diabetes mellitus (IDDM) in mice have been found to recognize an epitope shared by both mycobacterial and mammalian HSP60 (46). T cell lines isolated from experimental adjuvant arthritis in rats were found to recognize mycobacterial HSP65 (158). Of interest, immunization of rats with HSP65 did not lead to adjuvant arthritis but instead protected against it (159). Furthermore, immunization with mycobacterial HSP65 was found to protect Lewis rats against other forms of arthritis including, streptococcal cell wall-induced arthritis, collagen type II arthritis and arthritis caused by administration of the lipoidal amine CP20961 (160).

Attempts have been made to elucidate the mechanisms of protection by HSP65. For example, analysis of mycobacterial HSP65 epitopes involved in adjuvant arthritis demonstrated that HSP65 preimmunization did not down-regulate responses to the arthritogenic epitope 180-188 (see below). Rather, immunization enhanced responses to several other HSP65 epitopes that are not recognized in animals subjected to the protocol used to induce arthritis (161). Furthermore, a T cell line (A2) generated by immunizing Lewis rats with *M. tuberculosis* was found to induce arthritis when transferred to naive irradiated animals.

Two clones derived from this T cell line were found to either induce (clone A2b) or protect (clone A2c) against arthritis after adoptive transfer into Lewis rats. Furthermore, both clones were found to recognize epitope (a.a.180-188) of mycobacterium HSP65 and both utilized identical TCR  $\alpha$  and  $\beta$  chains. Analysis of cytokine production revealed that clone A2c produced 10-fold higher amounts of IFN- $\gamma$  than did clone A2b and that A2c proliferated in the presence of IFN- $\gamma$  while A2b was inhibited (162). These findings indicate that A2b (inducer) and A2c (protective) are respectively Th2 and Th1-like clones. This was opposite to what was expected since Th2 and Th1 cells are generally associated respectively with attenuation or exacerbation of inflammation and tissue damage (160).

Paradoxical findings involving recognition of HSP60 by the immune system have also been observed in other systems of experimental autoimmunity. For example, active immunization with a peptide designated p277 (a.a. 437-460) from human or murine HSP60 induces IDDM in NOD mice when the peptide is delivered conjugated to a carrier protein. Furthermore, adoptive transfer of T cell lines specific for peptide p277 induces diabetes in NOD mice or to naive C57BL/6 mice. However, administration with p277 without conjugation to a carrier prevents the development of IDDM in NOD mice and even reverses the course of overt disease in these animals (163). These findings suggest that in this model the development of autoimmune disease is influenced significantly by the manner in which the immune system encounters antigen. These observations together with the fact that HSP60-specific T cells are present both in healthy and in newborn individuals have suggested the hypothesis that autoimmunity to HSP60 is a natural phenomenon that may mark both health and disease. Thus, HSP60 may either amplify or restrain inflammation depending on specific anti-idiotypic and other regulatory cells present in the system (163). While the presence of a regulatory system for reactivity to HSP60 remains to be characterized, it is clear that peptides of HSP60 recognized by T cells are potent immunogenic carriers that can transform poorly immunogenic molecules into highly immunogenic ones (123).



## 5. HSP60 and natural immunity

Mycobacterial HSP65 stimulates human THP1 cells to produce and secrete proinflammatory cytokines including TNF, IL-6 and IL-8 (164). Treatment of murine peritoneal macrophages with HSP60s from *L. pneumophila*, *M. leprae*, *M. bovis* BCG or *E. coli* GroEL, induces mRNA accumulation for IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, TNF $\alpha$ , and GM-CSF as well as IL-1 secretion (165). Furthermore, exposure of murine peritoneal macrophages to mycobacterial HSP65 stimulates the release of the proinflammatory cytokines TNF- $\alpha$  and IL-6 and also induces production of reactive nitrogen intermediates (RNI). In addition, in vitro stimulation of *T. gondii* infected macrophages with mycobacterial HSP60 inhibits proliferation of the intracellular parasites (166). These findings suggest that HSP60 might be involved in initiating inflammation and granuloma formation during chronic intracellular infection via macrophage activation (164).

## 6. HSP60 as a protective antigen against intracellular pathogens

There is a substantial body of evidence indicating that HSP60 can elicit protective immune responses against intracellular infection in animal models. In the guinea pig, 86% of animals immunized with *L. pneumophila* HSP60 develop a strong cell mediated immune response and this correlates with protection against challenge with a lethal dose of bacteria (167). Similarly, HSP65 was found to be induced in peritoneal macrophages taken from mice immunized with a toxoplasma cell homogenate. The induction of this protein correlates with both increased numbers of  $\gamma/\delta$  T cells and protection against *T. gondii* infection and mice depleted of  $\gamma/\delta$  T cells die more frequently and more rapidly than do mice depleted of  $\alpha/\beta$  T cells (168,169). HSP60-specific CD4+  $\alpha/\beta$  T cell clones isolated from spleens of mice immunized with *Y. enterocolitica* HSP60 provide protection against infection with *Y. enterocolitica* when adoptively transferred into naive C57BL/6 mice (164). Similarly, J774 cells transfected with a *M. leprae* HSP60 cDNA and introduced into syngeneic Balb/c mice confer a high degree of protection against subsequent challenge with *M. bovis* or *M. tuberculosis*. Furthermore, adoptive transfer of HSP60-specific T cell clones isolated from *M. tuberculosis* immunized

mice, demonstrated that  $CD8^{+}\alpha/\beta$  T cell clones are the most effective in conferring protection (170). Orogastric immunization with recombinant GroES- and GroEL-like proteins from *H. pylori*, one of the causative agents of gastroduodenal infections, protects 80% of mice against helicobacter infection (171). Interesting, immunization with recombinant HSP70 from *Histoplasma capsulatum* significantly protects Balb/c mice against lethal intranasal challenge with virulent yeast (172). These findings strongly suggest that HSP60 T cell epitopes are recognized during intracellular infection and may confer significant levels of protection.

## 7. Summary

The HSP60 family are highly immunogenic molecules recognized by both B and T cells. Antibodies against HSP60 have been identified in a variety of human infections that involve either bacteria, protozoa or metazoa. B cell epitope mapping of HSP60 from *M. tuberculosis*, *M. leprae* and GroEL has demonstrated that antibodies against HSP60 (i) recognize epitopes distributed throughout the whole sequence of the protein, (ii) recognize linear determinants, and (iii) are either species-specific or cross-reactive. Similarly, T cells with specificity for HSP60 have also been identified both in the mouse and in humans. Mycobacterial HSP65 is currently the best characterized model. In mice immunized with *M. tuberculosis*, ~ 10-20% of peripheral T cells respond to mycobacterial HSP65. Approximately twelve human T cell epitopes have been mapped to mycobacterial HSP65. Furthermore, T cells specific for mycobacterial HSP65 have also been identified in healthy individuals including  $\gamma/\delta$  T cells that recognize HSP65 during infection and in healthy, PPD reactive individuals. Recognition of HSP60 by both  $\alpha/\beta$  and  $\gamma/\delta$  T cells from healthy individuals has led to the suggestion that these cells carry out an immune surveillance function.

HSP60 has also been implicated in various forms of autoimmunity. This view is supported by the presence of both  $\alpha/\beta$  and  $\gamma/\delta$  HSP60-specific T cells in individuals with different forms of autoimmune diseases. Some of these T cells recognize "self" epitopes in HSP60. Such T cell epitopes have been described in various autoimmune disorders including rheumatoid arthritis, Behcet's disease, arthritis induced by yersinia infection and juvenile chronic arthritis.

Furthermore, in the Lewis rat model of adjuvant arthritis, adoptive transfer of HSP60-specific T cells induces arthritis in naive recipients. However, attempts to induce arthritis by immunizing rats with HSP65 in the presence of adjuvant have not been successful. On the contrary, immunization with HSP65 induces protection against both adjuvant arthritis as well as other forms of arthritis including streptococcal cell wall, collagen type II and arthritis caused by administration of the lipoidal amine CP20961. It is evident, therefore, that reactivity to self-epitopes in HSP60 does not necessarily lead to development of disease. In fact, either self or foreign HSP60 T cell epitopes have been used as “carriers” or “adjuvants” in immunization studies to increase the immunogenicity of poorly immunogenic molecules without inducing autoimmunity.

Similarly, HSP60 has been found to be a protective antigen in animal models. Immunization with HSP60 protects mice against infection with *Y. enterocolitica*, *M. leprae*, *M. tuberculosis*, *H. pylori* and *H. capsulatum* and guinea pigs are protected against infection with *L. pneumophila*. In most of these models protection is T cell mediated. Furthermore, recent studies suggest that HSP60 may be involved in innate host responses in that macrophages respond to HSP60 from different bacteria including GroEL with the production of proinflammatory cytokines and ROI.

## **B. LEISHMANIASES**

### **1. Spectrum of disease**

The leishmaniasis comprise a group of diseases caused by parasites belonging to the genus *Leishmania*. Disease in humans presents a wide clinical spectrum and it is believed that this is in part dependent on the parasite species and the genetic background of the host (173). Four major clinical syndromes including visceral leishmaniasis, cutaneous leishmaniasis, mucocutaneous leishmaniasis and diffuse cutaneous leishmaniasis are seen most commonly (174).

Visceral leishmaniasis (VL) is mostly caused by *L. donovani* spp and occurs in many parts of the World including, India, Africa, the Mediterranean littoral, Asia, the Near East, and

South America. After infection, parasites disseminate throughout the body and are taken up by phagocytic cells in lymph nodes, liver, spleen and bone marrow. Classic VL is characterized by fever and by hepatosplenomegaly, leukopenia, hypergammaglobulinemia, thrombocytopenia, coagulation abnormalities, hemorrhage and bacterial sepsis. Death is a frequent feature of this disease (174).

Old and New World cutaneous leishmaniasis are caused respectively by *L. major* or *L. tropica* and *L. mexicana* and *L. braziliensis ssp.*. However, the basic pathogenesis for these diseases appears to be similar. After infection most parasites are likely killed with few required to establish disease. Both polymorphonuclear leukocytes and lymphocytes migrate to the site of initial parasite inoculation. This results in an erythematous pruritic papule and granulomatous skin lesion. In chronic lesions epithelioid cells and Langerhans type giant cells accumulate. Ulceration most probably results from a combination of factors that include edema, cellular infiltration and vasculitis leading to poor blood supply and necrosis. With an appropriate immune response or after successful chemotherapy, fibroblast activity becomes apparent and granulation tissue appears leading to healing with scar formation (174). Most individuals may resolve infection without apparent disease. This is supported by observations indicating that in endemic areas it is usual to find positive skin test responses to leishmania antigens without apparent lesions or scars.

*Leishmania (Viannia) spp.* are the causative agents of cutaneous (CL) and mucocutaneous leishmaniasis (MCL) in the New World. The latter is characterized by metastases, chronicity and recurrences of lesions following healing. In MCL, metastatic lesions frequently involve the formation of granulomatous lesions of the nose and oropharynx (175-177). Patients with MCL show high levels of delayed type hypersensitivity (DTH), lymphocyte proliferation and antibody titers. It has been suggested that the "reactogenicity," or sensitizing capacity of the leishmania involved may contribute to pathogenesis (176). Significant immune reactivity also correlates with low parasite loads at the site of infection (176,178). Parasite loads in tissue taken from patients or from infected hamsters have been found to decrease with the duration of

infection (179-181). Thus, the immune response clearly exerts substantial control over infection with these leishmania.

## 2. Leishmania invasion

*Leishmania* are dimorphic organisms. Part of the life cycle involves flagellated promastigotes in the gut of sandfly vectors, either *Phlebotomus* (in the Old World) or *Lutzomyia* (in the New World). Nonflagellated amastigotes reside inside phagolysosomes of macrophages in the vertebrate host (173).

Following an infected blood meal, the aflagellated amastigotes ( $\sim 3 \mu\text{m}$ ) enter the midgut of the female sandfly where they attach to the epithelium and transform into promastigotes ( $\sim 12 \mu\text{m}$ ) known as *procyclics*. As the parasites migrate forward to the proboscis of the sandfly, they become smaller (body length  $< 9 \mu\text{m}$ ) and move freely within the lumen of the intestine with the aid of a flagellum. At this stage they are referred to as *metacyclics* (173). The process of metacyclogenesis involves biochemical modifications such as changes in the major surface glycoconjugate lipophosphoglycan (LPG). Changes in LPG include capping of terminal disaccharides and elongation of the molecule resulting in a thicker structure that (i) serves as the acceptor molecule for complement, with mostly iC3b being the major fixation product, and (ii) confers resistance to the lytic action of the complement (182). Similar changes have also been found to occur when leishmania are cultured in medium where metacyclics are generated at stationary growth phase, that resemble the metacyclics found in the proboscis of the sandfly. Furthermore, stationary phase metacyclics are more infective for either mice in vivo or macrophages in vitro and are also more resistant to the toxic action of  $\text{H}_2\text{O}_2$  than are the forms found in log phase culture (183-185).

The infected sandfly inoculates metacyclic promastigotes while feeding on the mammalian host most likely by regurgitation (183). Parasites are taken up by macrophages in a process of receptor-mediated endocytosis that involves mainly CR1/CR3, mannose/fucose (MFR), fibronectin and Fc (FcR) receptors (173,186,187). Promastigote ligands include the leishmania surface metalloproteinase gp63 and LPG both of which bind to CR3 (173). While

CR1- mediated phagocytosis provokes less of an oxidative burst, thereby favoring intracellular survival, the MFR and FcR elicit strong oxidative burst activity and may promote parasite killing (187).

Once ingested by macrophages, promastigotes localize in phagosomes which fuse with secondary lysosomes to form a parasitophorous vacuole. In this environment of low pH (~ 4.5-6.0) and elevated temperature (32-37<sup>0</sup> C) metacyclics transform into amastigotes and become metabolically more active. Amastigotes divide and when the infected cell ruptures, they are released to infect other phagocytes. Enzymatic essays demonstrate that amastigotes (i) express a gp63-related metalloproteinase with optimal activity at pH (5.5-6.0), (ii) catabolize glucose, proline, and nucleosides at optimal pH of 4.5-5.0, and (iii) transport proline at a pH optima of 5.5 (188). Furthermore, LPG which is abundant in metacyclics is shed into the medium during transformation of promastigotes into amastigotes. Released LPG may (i) neutralize ROI by its ability to scavenge oxidants (189), (ii) inhibit protein kinase C and intracellular signaling and this may attenuate the macrophage oxidative burst (185,190). In support of the view that *Leishmania* may deactivate macrophages are the findings that amastigotes of *L. donovani* inhibit macrophage interleukin-1 (IL-1) responses and responses to IFN- $\gamma$  for induction of MHC class II molecules (191,192). Interestingly, MHC class II molecules have been localized in the parasitophorous vacuole of murine macrophages infected with *L. amazonensis* (193).

### **3. Immunity to *Leishmania***

#### **a. Antibodies versus T cells**

The host immune response to leishmaniasis involves both humoral and cell mediated immune responses. In vitro anti-leishmanial antibodies have been shown to promote phagocytosis and to lyse promastigotes in the presence of complement (194). There is no evidence, however, that antibodies play a protective role in the resolution of leishmania infection. Thus, high antibody levels have been noted in chronic infections, while low levels are associated with healing (195). In addition, transfer of immune serum does not provide

protection in experimental leishmaniasis (195). On the other hand, studies in mice demonstrate that cell mediated immunity (CMI) profoundly affects the outcome of infection. For example, CBA and C57BL/6 mice are highly resistant to *L. major* infection, whereas nude mice of these strains are unable to control infection. Resistance in nude mice can, however, be fully reconstituted with normal syngeneic T cells. Furthermore, resistance to *L. major* and *L. donovani* can be transferred by T cells, but not B cells (194-196).

#### **b. CD4 versus CD8 T cells**

CD8<sup>+</sup> T cells have been shown to be involved in protection against *Leishmania*. For example, depletion of CD8<sup>+</sup> cells by treatment with anti-Ly2 mAb results in delayed healing of otherwise resistant mice infected with *L. major* (197). Treatment of Balb/c mice with anti-Ly2 also abrogates protective immunity induced in these mice by intravenous immunization with irradiated promastigotes (198). More recently, it has been shown that CD8<sup>+</sup> cells may play an important role in immunity to reinfection with *Leishmania*. Up to a 50-fold increase in parasite-specific CD8<sup>+</sup> T cells were found in the spleens and lymph node cells of CBA or Balb/c mice reinfected with *L. major*. These CD8<sup>+</sup> T cells secreted significant amounts of IFN- $\gamma$  upon restimulation in vitro (199). Most experimental evidence suggests, however, that CD4<sup>+</sup> T cells are the primary class of T cells involved in resistance to leishmania infection. For example, transfer of Ly1<sup>+</sup> cells into nude mice renders these mice resistant to *L. major* and depletion of CD4<sup>+</sup> cells by in vivo treatment with anti-L3T4 mAbs leads to loss of resistance in otherwise resistant CBA mice (195). Furthermore, MHC class II gene knockout mice suffer fatal, uncontrolled infection with *L. major*, while  $\beta$ 2-microglobulin knockout mice with deficient MHC class I expression, contain infection in a manner not different from control mice (182).

#### **c. Th1 and Th2 cells**

Two subsets of CD4<sup>+</sup> cells designated Th1 and Th2 have been defined in mice based upon the lymphokines they produce which differentially affect macrophage activation. Th1

clones produce predominantly interleukin 2 (IL-2) and IFN- $\gamma$  and are host protective in leishmaniasis. Th2 cells produce IL-4, IL-5, and IL-10 and are disease-promoting (200). Th1 cells mediate resistance through the production of IFN- $\gamma$  which activates macrophages to kill leishmania. On the other hand, Th2 cells and the lymphokines they produce are associated with enhanced humoral immunity (particularly the IgE mediated immune response) and inhibition of macrophage activation (185,199). Recent studies indicate that similar cytokine patterns and their effects appear to contribute to the pathogenesis of human leishmaniasis (201).

Differential functional properties of these CD4<sup>+</sup> T cell subsets in leishmaniasis have been confirmed in vivo. For example, T cells obtained from susceptible Balb/c mice infected with *L. major* have been found to contain mainly CD4<sup>+</sup> T cells expressing IL-4 transcripts and secreting IL-4. In contrast, CD4<sup>+</sup> T cells from the resistant C57BL/6 mice express transcripts for IFN- $\gamma$  and secrete IFN- $\gamma$ , but not IL-4 (202). Furthermore, adoptive transfer of Th1 T cell clones from leishmania infected mice into susceptible, naive Balb/c mice confers resistance to infection, whereas transfer of Th2 clones into resistant CBA mice exacerbates disease (182).

#### **d. Activation of macrophages**

Cytokines produced by Th1 cells are involved in the regulation of macrophage activation and subsequent control of leishmania infection. In this regard, IFN- $\gamma$  has been found to be critical to disease control. Resistant strains of mice lacking either IFN- $\gamma$  or IFN- $\gamma$  receptor genes are unable to control *L. major* infection. Furthermore, in vitro experiments have shown that macrophages (derived from either susceptible or resistant mice) when they are stimulated with recombinant IFN- $\gamma$  are able to clear intracellular amastigotes (182). However, other cytokines may interact with IFN- $\gamma$  for maximal activity. Thus, in murine peritoneal macrophages IFN- $\gamma$  synergizes with granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-7 or IL-4 to enhance resistance to leishmania infection in vitro. Furthermore, killing of amastigotes by murine peritoneal macrophages is dramatically enhanced when these cells are incubated with IFN- $\gamma$  plus simultaneous administration of either IL-4 or TNF- $\alpha$ .



Administration of  $\text{TNF-}\alpha$  alone is either ineffective or induces only slight antimicrobial activity, whereas treatment of macrophages with IL-4 before stimulation with  $\text{IFN-}\gamma$  inhibits the ability of macrophages to kill intracellular *L. major* (185).

#### **e. Killing of intracellular *Leishmania***

The mechanisms by which macrophages destroy intracellular leishmania have been studied. Although evidence indicates that ROI contribute to killing, most studies suggest that NO constitutes the major effector molecule. Thus, it has been shown that killing of *L. major* promastigotes or amastigotes correlates with enhanced synthesis of NO by macrophages (182). Conversely, control of infection is abrogated either when resistant mice in vivo or infected macrophages in vitro are treated with  $\text{N}^{\text{G}}$ -monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthesis. Furthermore, intralesional application of L-NMMA results in exacerbation of disease in resistant mice (182,185). Consistent with a major activating role for  $\text{IFN-}\gamma$  are findings demonstrating that  $\text{IFN-}\gamma$  alone is capable of enhancing iNOS mRNA and NO release from murine peritoneal macrophages and that  $\text{TNF-}\alpha$  synergizes with  $\text{IFN-}\gamma$  to induce iNOS activity and NO production by these cells (182). Although NO has been shown to be a key factor in intracellular killing of leishmania by murine macrophages, it may not play the same role in human macrophages. This is thought to be due to the fact that human monocyte/macrophages fail to produce or produce only limited amounts of NO upon stimulation by either  $\text{IFN-}\gamma$ , tumor necrosis factor  $\alpha$  ( $\text{TNF-}\alpha$ ) or LPS. On the contrary, stimulation of murine macrophages with the same cytokines results in rapid and abundant production of NO. This dichotomy appears to be explained by differences in the 5' regulatory regions of the murine and human NO synthase genes (201a).

#### **f. Generation and role of Th1 and Th2 cells**

Recently, a substantial body of research has been concerned with identifying the mechanisms and cytokines involved in the differentiation of precursor T helper Th0 cells into either Th1 or Th2 cells. Although the data suggest that certain antigens may provoke a

particular type of Th responses, other findings indicate that naive Th0 cells have the potential to differentiate into either Th1 or Th2 cells and that the types of cytokines present at the time of primary antigen stimulation are critical for determining outcome (182,199). For example, naive CD4<sup>+</sup> T cells from transgenic mice expressing T cell receptors (TCR) specific for cytochrome C in the context of MHC class II, cultured in the presence of IL-12 and antigen, develop into Th1 cells producing IFN- $\gamma$  upon restimulation. Administration of IL-4 to these IL-12-treated cells reduces, but does not abolish IFN- $\gamma$  production. Furthermore, addition of anti-IL-4 antibody to the primary culture to neutralize the endogenous IL-4, results in the generation of T cells producing IFN- $\gamma$  and IL-2 upon restimulation (203). In another system, CD4<sup>+</sup> T cells isolated from mice immunized with cytochrome C and restimulated in vitro with antigen presenting cells (APC), cytochrome C and IL-4 differentiated into IL-4 producing cells (204,205). Similarly, naive CD4<sup>+</sup> murine T cells cultured in vitro with either anti-CD3 mAb, Con A, or alloantigen in the presence of IL-4, show increased IL-4 production and markedly decreased synthesis of IL-2 and IFN- $\gamma$ . Also, when stimulated with IL-12 and either Con A or PHA, human umbilical cord blood CD4<sup>+</sup> T cells produce large amounts of IFN- $\gamma$ , but not IL-4 in vitro (206).

Similarly, in experimental leishmaniasis, CD4<sup>+</sup> lymph node T cells from both susceptible and resistant mice infected with *L. major* show mixed expression of cytokines including IL-2, IL-4 and IL-13 that peaks on day four. Expression of IFN- $\gamma$  by CD4<sup>+</sup> cells is more variable among various strains of mice with expression being predominantly by NK cells (182). Remarkably, increased levels of IL-4 are observed in all mouse strains and are highest in Balb/c mice. However, resistant mice rapidly downregulate IL-4 expression, whereas susceptible Balb/c continue to express high levels (182).

Susceptible Balb/c mice infected with *L. major* and treated immediately with IL-12 showed a remarkable reduction in parasite burden and a cure rate of 89%. When lymph node cells from IL-12 treated mice are restimulated in vitro with leishmania antigens, they produce significantly less IL-4 and more IFN- $\gamma$  when compared to CD4<sup>+</sup> T cells from infected mice that had not received IL-12. Furthermore, when IL-12 recipients are also treated with anti-

IFN- $\gamma$  antibodies, partial abrogation of the IL-12 curative response occurs and this correlates with restoration of a Th2 cytokine response in vitro (207). Consistent with the protective and disease promoting effects of respectively IL-12 and IL-4, are findings that treatment of resistant C57BL/6 mice with anti-IL-12 mAb results in more severe disease which is reversed by administration of anti-IL-4 mAb. These findings indicate that endogenous IL-12 may be required to control a Th2 response in leishmania resistant mice (208). Taken together, these results suggest that IL-12 is a dominant factor leading to Th1 cell development, whereas IL-4 may be responsible for the emergence of Th2 cells.

### **f.1. Interleukin 12 (IL-12)**

IL-12 is a heterodimer composed of two polypeptides with sizes of 35-kDa (p35) and 40-kDa (p40) in both human and the mouse. The IL-12 receptor is a member of the hematopoietin receptor superfamily that appears to be specific for IL-12 since it does not respond to a variety of other cytokines. Since antibodies specific to p40 inhibit binding of IL-12 to the its receptor, it has been suggested that p40 is the receptor binding subunit (209). IL-12 was initially characterized for its ability to activate NK cells to kill tumor target cells and virus infected cells (209). In conjunction with IL-2, IL-12 activates cytotoxic T lymphocytes (CTL) to kill allogenic melanoma tumor cells (209).

Although IL-12 and free p40 are produced by some transformed B cell lines following stimulation with phorbol ester and by keratinocytes and dendritic cells, IL-12 is produced primarily by cells of the monocyte/macrophage lineage in response to bacteria, bacterial products and intracellular parasites (209-211). In fact, highly purified macrophages and heat-killed *L. monocytogens* (HKLM) prime and stimulate naive, ovalbumin-specific alpha beta TCR transgenic T cells to become IFN- $\gamma$  producers upon restimulation with OVA. The effect of HKLM is not due to enhanced antigen-presentation since increased priming for the production of IFN- $\gamma$  is seen even if the macrophages used express class II MHC molecules not recognized by the responding transgenic T cells. Priming for IFN- $\gamma$  production is inhibited by anti-IL12 antibodies and when IL-12 is used instead of HKLM or when different APCs are

used together with IL-12, the transgenic T cells invariably produced IFN- $\gamma$  (184,204). These findings are consistent with the view that macrophage production of IL-12 plays a role in enhancing priming for IFN- $\gamma$  production.

Similar results to these have also been obtained in murine leishmaniasis. For example, two days after infection of resistant C3H mice with *L. major*, high levels of both IL-12 mRNA and IL-12 producing cells are found in the lymph nodes (199). In another study, increased numbers of IL-12 producing cells and IL-12 production were detected as early as 1 day after infection of C3H mice with *L. major* (212). These and other studies suggest that IL-12 is produced by macrophages in response to leishmania infection and that IL-12 (i) primes naive Th0 cells to differentiate into Th1 cells, and (ii) stimulates NK cells to produce IFN- $\gamma$ . Thus, IL-12 appears to promote CMI and to link innate immunity with the adaptive immune response (210).

## **f.2. Role of NK cells**

It has been suggested that IL-12 may contribute to control of leishmania infection in part by activating NK cells to produce IFN- $\gamma$  which subsequently promotes Th1 cell development (199). In support of this view are findings indicating that activation of NK cells by in vivo administration of IL-12 facilitates Th1 cell development and protection of Balb/c mice against *L. major* infection (213). In this study, IFN- $\gamma$  production by lymph node cells was found to correlate with NK cell lytic activity of target cells. In addition, depletion of NK cells in vivo prior to administration of IL-12 resulted in reduction of IFN- $\gamma$  production by both lymph node and spleen cells in vitro (213). In resistant mice, both IFN- $\gamma$  production and NK cell lytic activity is dependent upon the presence of IL-12 since treatment of leishmania infected C3H mice with anti-IL-12 mAb suppresses early NK cell lytic activity, diminishes IFN- $\gamma$  production and promotes disease development (199,213). In other study, administration of IL-12 prior to infection of either C57BL/6, CBA or C3H/HeJ mice prevented early dissemination of parasites into visceral organs. Conversely, parasites spread rapidly when C57BL/6 mice were depleted of NK cells or treated with anti-IFN- $\gamma$  prior to infection.

Furthermore, the ability of naive T cells to differentiate into Th1 cells after transfer into *L. major*-infected SCID mice may be attributed to the production of IFN- $\gamma$  by NK cells (199). As discussed below, Th2 type cytokines may down regulate IL-12-mediated activation of NK cells in Balb/c mice and this may increase susceptibility to *L. major* infection.

### **f.3. Roles and sources of IL-4**

The role of IL-4 in priming for Th2-like responses in leishmaniasis is supported by several lines of evidence. For example, treatment of Balb/c mice infected with *L. major* with anti-IL-4 antibodies significantly attenuates disease. Furthermore, T cell clones derived from such mice treated with anti-IL-4 antibodies preferentially produce IFN- $\gamma$  whereas in the absence of antibody treatment these clones produce mainly IL-4. Despite results such as these indicating that IL-4 may be responsible for disease progression in Balb/c mice, recent research suggests that IL-4 may not be the sole determinant of murine susceptibility to *L. major*. Thus, disruption of the IL-4 gene does not lead to a reduction in either lesion size or parasite loads in Balb/c mice infected with *L. major*. Furthermore, analysis of cytokine transcripts and cytokine secretion from lymph node cells of infected, IL-4 knockout mice indicates that the typical Th2 pattern of cytokines induced by leishmania infection in normal Balb/c mice is unaltered (214).

While macrophages appear to be the primary source of IL-12 in the case of Th1 cell development, the origin of IL-4 is less clear. Two candidates for IL-4 production include: (i) CD4<sup>+</sup>, NK1.1<sup>+</sup> cells found in the thymus and bone marrow, that produce IL-4 or IL-4 and IFN- $\gamma$  upon stimulation without the need for previous activation, and (ii) basophils and mast cells which secrete IL-4 upon cross-linking of Fc $\epsilon$ RI or Fc $\gamma$ RII/III receptors. Presently, the origin of IL-4 during leishmania infection remains unresolved (204).

### **f.4. Role of IFN- $\gamma$**

Whether IFN- $\gamma$  is directly involved in priming and differentiation of CD4<sup>+</sup> T cells into Th1 cells is controversial (204). Results both supporting and refuting this possibility have

been obtained. For example, addition of IFN- $\gamma$  to naive CD4<sup>+</sup> T cells in the presence of Con A and APCs enhances IFN- $\gamma$  production, consistent with a Th1 response (204). In contrast, addition of IFN- $\gamma$  to primary cultures of TCR transgenic T cells specific for pigeon cytochrome C does not enhance priming for IFN- $\gamma$  production (204). In murine leishmaniasis, neither sustained delivery of IFN- $\gamma$  for up to 6 days post infection nor infection with promastigotes of *L. major* expressing the IFN- $\gamma$  gene alters disease progression in susceptible Balb/c mice (182). However, treatment of resistant C3H/HeN or C57BL/6 mice with anti-IFN- $\gamma$  antibodies at the time of infection with *L. major* results in Th2-type cytokine production and more severe disease. Similarly, disruption of the IFN- $\gamma$  gene in C57BL/6 mice leads to failure to control disease and CD4<sup>+</sup> T cells expressing a Th2 phenotype (182,199). Taken together, these results suggest that IFN- $\gamma$  may be necessary, but not sufficient for the induction and maintenance of a Th1 type response. The exact mechanism by which IFN- $\gamma$  supports development of a Th1 response is not clear. This may involve either a direct effect on differentiation of Th0 cells or inhibition of IL-4 production which normally inhibits priming for development of Th1 cells (204).

#### **f.5. Other cytokines**

Cytokines other than IL-12, IFN- $\gamma$  or IL-4 may influence the differentiation of Th0 cells into either Th1 or Th2 effectors. Lymph node cells from Balb/c mice infected with *L. major* taken 2 days after infection produce IFN- $\gamma$  (derived from NK cells), IL-4, IL-10 and TGF- $\beta$  upon restimulation in vitro. Simultaneous neutralization of IL-4, IL-10 and TGF- $\beta$  using specific antibodies maximizes IFN- $\gamma$  production under these conditions. Neutralization of TGF- $\beta$  alone results in significantly enhanced IFN- $\gamma$  synthesis. Inhibition of the production of IFN- $\gamma$  by IL-4, IL-10 and particularly TGF- $\beta$  may contribute to disease progression by promoting Th2 cell development (199). Supporting this possibility are findings indicating that treatment of C57BL/6 mice with TGF- $\beta$  prior to infection with *L. amazonensis* or *L. braziliensis* results in more severe disease and in increased IL-4 transcripts in lymph node cells (215).

IL-2 is critical for priming of both Th1 and Th2 cells since this cytokine's most important effect is to act as growth factor. In murine leishmaniasis, it has been reported that Balb/c mice infected with *L. major* and treated with anti-IL-2 antibody have diminished IL-4 production and the majority of mice heal their infection in contrast to untreated mice that have progressive disease (216).

#### **f.6. Influence of genetic background**

Studies in transgenic mice have shown that the genetic environment in which responder T cells reside may influence the development of Th1 or Th2 cell phenotypes. For example, when T cells from (*L. major*-susceptible) Balb/c transgenic mice expressing a TCR specific for a chicken ovalbumin peptide (cOVA) are cultured in vitro with syngeneic APCs and OVA (without added cytokines) they produce large amounts of IL-4 and little IFN- $\gamma$ . The outcome is reversed when T cells from (*L. major*-resistant) transgenic B10.D2 mice (B10 mice containing the Balb/c H-2 locus) also expressing cOVA-specific TCR, are cultured under the same conditions. In contrast, T cells from both transgenic strains develop into either Th1 or Th2 phenotypes when exposed respectively to either IL-12 or IL-4 in vitro during primary stimulation. Furthermore, when T cells from these mice are initially stimulated under neutral conditions (irradiated syngeneic APCs plus OVA peptide and no added cytokines) and then restimulated with IL-12 and TA3 APC (which produce neither IL-12 nor IFN- $\gamma$ ) Balb/c T cells do not respond to IL-12 in the second stimulation in terms of increased IFN- $\gamma$  synthesis, whereas B10.D2 show 10-fold increases in IFN- $\gamma$  production. These findings suggest that the susceptibility of Balb/c mice to infection with *L. major* may be due to a genetically determined inability to generate an IL-12-induced response rather than from a dominant IL-4 T cell response (217,218).

#### **f.7. Importance of antigens**

Treatment of Balb/c mice with anti-IL-4 antibodies prior to infection with *L. major* results in a predominant Th1 response in contrast to untreated, control mice where the response to *L.*

*major* involves predominantly Th2 cells. Analysis of TCR usage by CD4<sup>+</sup> T cells from these two groups of mice demonstrates that both expand Th1 and Th2 cells with restricted clonotypes bearing TCRs using V $\beta$ 4/V $\alpha$ 8 heterodimers. Furthermore, protective Th1 cell clone (9.1-2) derived from a Balb/c mouse immunized with parasite antigens was found to use a similar TCR (219). These findings, together with the results indicating that it is possible to convert susceptible Balb/c mice into a resistant phenotype using immunological interventions, suggests that parasite antigens that drive CD4<sup>+</sup> T cell responses may not be the major determinants for Th1 versus Th2 cell development (182,219). Nevertheless, evidence indicates that antigens eliciting specific T cell subtypes that contribute to resistance or to disease susceptibility exist. For example, both Balb/c and B10.D2 mice immunized with a peptide (p183) derived from a protein of *L. major* have significant disease exacerbation upon challenge with *L. major* promastigotes. Furthermore, lymph node cells isolated from p183 immunized Balb/c proliferate and secrete IL-4, but not IFN- $\gamma$  upon stimulation in vitro with p183 (220). Conversely, antigens eliciting Th1 type responses that confer protection upon immunization have also been characterized (see below).

#### **f.8. Modulation of Th1 and Th2 cell development**

Elucidation of the mechanisms and elements -such as IL-4 and IL-12- that influence the generation of Th1 and Th2 cells opens the possibility for immunological interventions aimed at driving the immune response in an specific direction. For example, when highly polarized Th1 cells from Balb/c mice infected with *L. major* are cultured in the presence of *L. major* antigen, IL-4, APC, and IL-2, increased levels of IL-4 and IL-10 are produced upon restimulation with leishmania antigen alone. Similarly, a Th2 cell population cultured in the presence of IL-12, IFN- $\gamma$ , IL-2, APC and anti-IL-4 Abs shows substantially increased IFN- $\gamma$  production upon subsequent stimulation with antigen alone (221). The ability of IL-12 to influence the induction of Th1 type cells has also been used as a strategy for vaccination. When used as adjuvant, IL-12 in combination with leishmania antigens (see below) protects Balb/c mice against infection with *L. major* (213).



#### 4. Vaccine development against *Leishmania*

As indicated above, the roles for individual antigens in driving a particular Th pattern are controversial (219). However, it is clear that protective leishmania antigens do exist (222-224). Both crude preparations and purified proteins have been used in immunization studies in mice with protocols that include adjuvants targeting Th1 type responses.

The quantity of antigen used for immunization may be critical for the development of an effective Th1 type immune response. For example, Balb/c mice immunized with low numbers of promastigotes (between  $10^3$  and  $3.3 \times 10^3$ ) develop increased IgG2a antibodies consistent with a Th1 response and resistance to infection with *L. major*. In contrast, mice immunized with larger numbers of promastigotes develop IgG1 antibodies and more severe infection (225).

Data from murine models indicate that the route of antigen administration as well as the antigen used for immunization influences the responses of  $CD4^+$  cell subsets. For example, Balb/c mice immunized intravenously with whole lysates of promastigotes of *L. mexicana amazonensis* are protected against subsequent challenge with promastigotes for up to 6 months. They show both reduced lesion sizes and parasite numbers in the spleen as well as fewer metastases (226). Susceptible Balb/c mice are also protected against *L. major* infection by intravenous or intraperitoneal immunization with irradiated promastigotes while subcutaneous immunization leads to enhanced lesion development. This suggests that Th1 and Th2 cells are preferentially stimulated in response to respectively systemic or subcutaneous immunization (194,195). A soluble, membrane-free leishmania antigen (SLA), obtained from sonicated promastigote of *L. major* has also been studied as a vaccine candidate. Balb/c mice immunized intraperitoneally with 100 ug of SLA combined with *C. parvum* show ~ 89% protection (227). Partial purification of SLA, yields a fraction 9 that is recognized by Th1-protective cells and a fraction 1 that is recognized by Th2-disease promoting cells. Furthermore, a Th1 cell line and a Th1 clone derived from this line (both responsive to fraction 9) are each able to protect mice from infection with *L. major* (195).

The abundant leishmania surface protein gp63 has been extensively investigated as a vaccine candidate against leishmaniasis. When reconstituted into liposomes, gp63 significantly protects CBA/ca and Balb/c mice against *L. mexicana* (222). T cell epitope mapping of gp63 identified a peptide which when inoculated with adjuvant induces a Th1 response and confers protection against two species of *Leishmania* (228). Similarly, two T cell epitopes (p146-171 and p 467-171) from gp63 were found to induce a Th1 cell response and to protect Balb/c mice against *L. major* (229). Oral administration of *S. typhimurium* carrying a plasmid expressing gp63 induces a Th1 response and confers a high degree of protection against *L. major* infection in CBA mice (230). Significant protection against *L. major* infection is also obtained by immunizing Balb/c mice with BCG expressing gp63 (231).

Other protective antigens derived from *Leishmania* have been described. A 46-kDa membrane glycoprotein (M-2) of *L. amazonensis* when administered in combination with *C. parvum* as adjuvant confers complete protection to 28 to 50% of Balb/c mice (223). Vaccinia virus expressing M-2 also protects 45-70% of Balb/c mice against infection with *L. amazonensis* (232). A promastigote antigen-2 (PSA-2) comprised of three polypeptides of 96, 80 and 50 kDa (encoded by genes of the same family that encode M-2), has been prepared from promastigotes of *L. major*. Immunization of C3H/He mice with PSA-2 and *C. parvum* results in complete protection against *L. major* infection (233). A protein dp72 has been isolated from promastigotes of *L. donovani*. Immunization of Balb/c mice with dp72 and *C. parvum* as adjuvant reduces the number of parasites in the liver by more than 80% as compared to control mice immunized with *C. parvum* alone (234). Two antigens (P4 and P8) have also been immunoaffinity purified from amastigotes of *L. pifanoi*. Immunization with P4 and P8 in the presence of *C. parvum* as adjuvant results in protection of Balb/c mice against infection with promastigotes of *L. pifanoi* (235). A ~ 40 kDa antigen (Lcr1) obtained from a cDNA library of *L. chagasi* amastigotes was found to significantly protect Balb/c mice against challenge with *L. chagasi* promastigotes (236). Immunization with a crude preparation of proteins in the range of 64 to 97 kDa derived from either *L. infantum* or *L. major* in combination with muramyl dipeptide as adjuvant protects Balb/c mice against subsequent

infection with *L. infantum* (237). Also, a fraction of membrane proteins extracted from *L. major* with Triton X-114 significantly protects Balb/c mice against *L. major* infection (238).

Live leishmania vaccine preparations have also been found to effectively induce protection against infection. Immunization of Balb/c mice with an avirulent strain of *L. major* defective in the expression of lipophosphoglycan (LPG), induces Th1 cells. When these are adoptively transferred into naive Balb/c recipients significant protection against *L. major* is achieved (239). A *L. major* null mutant (dhfr-ts<sup>-</sup>) developed by deleting the dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) genes is unable to cause disease in Balb/c mice. However, when inoculated into Balb/c mice this mutant induces significant resistance to challenge with virulent *L. major* (240).

More recently, an immunization protocol using specific leishmania antigens in combination with IL-12 as adjuvant has been developed. Balb/c mice immunized with SLA and IL-12 develop Th1 cells and significant protection against infection (213). Similarly, a Th1 T cell clone obtained from mice immunized with SLA antigen identified a protein of 36 kDa (LACK) in a cDNA library. Balb/c mice immunized with Lack and IL-12, but not LACK alone are significantly protected against infection with *L. major* (241). These findings suggest that IL-12 may substitute for bacterial adjuvants in vaccine development.

## 5. Summary

The leishmaniasis are a group of diseases caused by protozoan parasites of the genus *Leishmania*. These dimorphic organisms live part of their life cycle in the alimentary tract of an insect vector which inoculates the parasite while feeding on the mammalian host. After being taken up by macrophages, the remainder of the life cycle is spent inside the phagolysosome of the macrophage in the amastigote form. The leishmaniasis in humans present with different clinical manifestations including visceral, cutaneous and mucocutaneous phenotypes. It is currently held that these diverse presentations are dependent both on the parasite species initiating the infection and on the genetic background of the host.

Resistance to leishmania infection involves the generation of a leishmania-specific T cell immune response which results in elimination of intracellular forms. In this regard, studies in mice have demonstrated the existence of 2 subsets of  $CD4^{+}$  T cells designated Th1 and Th2. Th1 cells are host protective in leishmaniasis producing predominantly IL-2 and IFN- $\gamma$ . These cytokines activate macrophages to produce NO which contributes to killing of intracellular *Leishmania*. Disease-promoting Th2 cells produce mainly IL-4, IL-5, and IL-10 which interfere with macrophage activation. Generation of Th1 cells requires the existence of a subset of naive  $CD4^{+}$  T cells which when stimulated with leishmania antigen in the presence of IL-12 and IFN- $\gamma$  develop a Th1 phenotype. On the other hand, if IL-4 is present during priming, the resulting  $CD4^{+}$  T cells may develop a Th2 phenotype. Other factors such as co-stimulatory cytokines, antigen, antigen presenting cells and accessory molecules may also influence the type of T cell response. However, the roles of these factors appears to be secondary to the dominant role of the priming cytokines, IL-12, IL-4 and IFN- $\gamma$ .

Immunization studies in mice have provided clear evidence that leishmania antigens when administered under conditions that promote a Th1 cell response may elicit protective immune responses against subsequent infection with *Leishmania*. This has been demonstrated with the leishmania antigens SLA, M-2, PSA-2 and gp63 each of which given in combination with *C. parvum* as adjuvant confers high levels of protection in susceptible Balb/c mice. The involvement of IL-12 in stimulation of cell mediated immunity, in activating NK cells and in inducing Th1 cells suggests that this cytokine may be a useful adjuvant. In fact, IL-12 in combination with the leishmania antigens SLA and LACK protects susceptible mice against subsequent infection with *Leishmania*.

## **D. LEISHMANIA HEAT SHOCK PROTEINS**

### **1. Heat shock proteins and leishmania infection**

*Leishmania* undergo an abrupt temperature change during transition from the insect vector (21 $^{\circ}$ -26 $^{\circ}$  C) to the mammalian host (37 $^{\circ}$  C). This dramatic change suggests that leishmania HSPs might be involved in survival of the parasite in the hostile environment of the host cell,

the macrophage. *Leishmania* promastigotes exposed to high temperatures or serum deprivation, show increased expression of HSP belonging to several families including HSP82, HSP70, HSP65, HSP41, HSP23 and HSP22 as determined by [ $^{35}\text{S}$ ] methionine labeling (242). A small 22-kDa HSP which localizes to the mitochondrion has been found to be induced in *L. mexicana amazonensis* (Lma) after exposure to heat shock at 37 $^{\circ}$  C (243).

When *L. mexicana* are cultured in medium at pH 5.5 a homogeneous stationary phase population of metacyclic forms develops (244). Furthermore, both low pH and elevated temperatures induce the transformation of promastigotes to amastigotes. This has been observed for diverse species of leishmania including *L. pifanoi*, *L. braziliensis*, *L. mexicana*, *L. donovani*, *L. infantum*, *L. major* and *L. tropica* (245). In addition, promastigotes of *L. braziliensis* exposed to 34 $^{\circ}$  C are more infective for syrian hamsters than are promastigotes cultured at 26 $^{\circ}$  C (246). Whether these profound changes are related to the expression of HSPs remains to be examined.

Stress proteins may help *Leishmania* cope with the toxic potential of ROI produced in the macrophage. Exposure of *L. donovani* promastigotes to either heat shock at 37 $^{\circ}$ C or to sublethal concentrations of H<sub>2</sub>O<sub>2</sub> induces expression of HSP70 and protects them against subsequent exposure to otherwise lethal concentrations of H<sub>2</sub>O<sub>2</sub> (184,247). In addition, treatment of *L. chagasi* promastigotes with sublethal concentrations of the oxidant menadione increases their virulence for Balb/c mice. Thus, animals infected with menadione-treated promastigotes show increased numbers of amastigotes in their livers as compared to animals infected with normal parasites. These results suggest that a cross-protective stress response occurs in promastigotes upon exposure to either heat shock or to oxidant stress (247).

Other studies indicate that while a wide range of HSPs are induced during heat shock of leishmania, expression of HSP70 and HSP83 are dominant phenomena (248,249). These proteins are found constitutively at high levels (2.1% and 2.8% respectively of total cell protein in *L. major*) and are induced further at 37 $^{\circ}$  C (250). Four *HSP70* genes are induced when *L. major* are exposed to heat shock at 37 $^{\circ}$  C. These genes are arranged in tandem repeats separated by intergenic regions of ~ 380 bp. A fifth identical *HSP70* gene which is

temperature independent is positioned in a separate locus (251). In another study, three independent genes related to the HSP70 family were found to be localized on different chromosomes of *L. major*. Two of these genes are constitutively expressed in promastigotes and the third is differentially expressed between procyclic and metacyclic forms (252). Recently, a *HSP70* gene containing an N-terminal sequence characteristic of a mitochondrial targeting signal has been isolated. This gene is expressed in all stages of *L. major* (253). A *HSP70* gene from *L. mexicana amazonensis* found to be encoded by a 24-kb genomic locus contains seven tandem repeats of 3.5 kb each and an additional *HSP70* gene positioned in a distinct locus. The predicted protein sequence of these Lma genes shows ~ 90% sequence identity with HSP70 from *L. donovani*, 81-85% with *T. cruzi* HSP70 and 68-72% with the human homolog (188). A cognate member of the *HSP70* gene from *L. donovani* was cloned from a *L. donovani* expression library using immune sera from patients with visceral leishmaniasis. This protein is recognized by sera from patients with visceral leishmaniasis, but not by sera from patients with cutaneous leishmaniasis (131). The leishmania *HSP70* mRNAs are processed by trans-splicing and have mini-exons joined to their 5' ends. The 3' end of the *HSP70* mRNAs consist of long untranslated tails (1.0 kb) containing repetitive sequences (251). Interestingly, the *HSP70* locus is not transcribed as a polycistronic pre-mRNA as is usually the case in other gene loci in trypanosomes. Rather the *HSP70* genes are transcribed individually with transcription termination occurring in the intergenic sequences (254).

HSP83 is localized in the cytoplasm of Lma. The *HSP83* gene has been cloned and found to be present in several copies of 4-kb repeats arranged in tandem on a chromosome of ~ 1Mb (255). Of interest, while HSP83 mRNA is rapidly degraded at 26<sup>o</sup> C, it becomes highly stable at 35<sup>o</sup> C (256). Furthermore, elevated temperatures do not increase transcription of the *HSP83* gene of Lma, even though exposure of promastigotes to 37<sup>o</sup> C results in increased expression of HSP83. This suggests that heat shock regulates the *HSP83* gene at the posttranscriptional level in Lma and that accumulation of mRNA at 37<sup>o</sup> C results mainly from increased stability of the mRNA at high temperature (256). Recent studies demonstrate that

the *HSP70* gene is also regulated primarily at a post-transcriptional level in both *L major* and *L donovani* suggesting that this may be a general feature in *Leishmania* (250).

In contrast to HSP70 and HSP 83, relatively little is known regarding the HSP60 family of proteins from *Leishmania*. Promastigotes of *L. major* treated with heat shock at 34<sup>0</sup> C show induction of several prominent proteins including a putative HSP60 as determined by [<sup>35</sup>S]methionine labeling (242). However, the regulation of HSP60 expression in *Leishmania* and the expression of this protein in leishmania-infected macrophages have not been addressed.

## 2. HSPs and the immune response to *Leishmania*

Recent data indicate that leishmania HSPs are antigenic in vivo and that the immune responses elicited are specific. An amastigote cDNA library from *L chagasi* was screened with sera pooled from patients with visceral leishmaniasis. Forty-six percent of a total of 242 positive clones were accounted for by either HSP70, HSP83 or HSP90 with the vast majority of these (86%) corresponding to HSP70. Up to 50% of serum samples obtained from patients with visceral leishmaniasis recognize HSP70 from *L. donovani* (131). This phenomenon may be specific in that sera from patients with either cutaneous leishmaniasis, Chagas' disease, leprosy, malaria or schistosomiasis fail to react with *L. donovani* HSP70 to a significant extent (131).

B cell epitope mapping of HSP70 using sera from patients with visceral leishmaniasis identified an immunodominant sequence located in the carboxy-terminal region, the most evolutionarily divergent region of the molecule. Of note, the sequence, EEDDKA, located at position 529-534 is not recognized by sera from patients with Chagas' disease, schistosomiasis, or cutaneous leishmaniasis(257). Similarly, sera from visceral leishmaniasis patients recognize both recombinant leishmania HSP70 and HSP90 while sera from Chagas' disease patients do not (257). Given that HSP70 and HSP90 from *Leishmania* ssp. share more than 80% amino acid identity with their counterparts in *T. cruzi*, selective recognition of individual epitopes by sera from patients with leishmaniasis indicates a high degree of specificity.

Only a limited amount of information is available regarding T cell responses to HSPs in the leishmaniasis. Peripheral blood mononuclear cells (PBMC) from patients with either CL or MCL have been used to characterize T cell responses to recombinant HSP83 and HSP70 of *L. braziliensis*. PBMC from all leishmania-infected individuals were found to proliferate and secrete IL-2, IFN- $\gamma$  and TNF- $\alpha$  in response to rLHSP83. On the other hand, rLHSP70 was observed to stimulate proliferation of PBMC from patients with MCL, but not CL. In contrast, PBMC from these MCL patients did not respond to recombinant human HSP70. This Th1-like cytokine response to HSP83, suggests that this leishmania HSP may be involved in protection rather than in disease promotion during both MCL and CL (258). This may also be true for leishmania HSP70 since recent research indicates that: (i) lymphocytes from asymptomatic patients with kala-azar proliferate *in vitro* and secrete IFN- $\gamma$  and IL-2 in response to rLHSP70 from *L. donovani chagasi* (Ldc), and (ii) seropositive, asymptomatic dogs have positive DTH reactions to rLHSP70 from Ldc (259).

Although it is clear that T cells are crucial for resolution of leishmania infection, relatively little is known about the nature and role played by  $\gamma/\delta$  T cells in the immune response to *Leishmania*.  $\gamma/\delta$  T cell numbers are increased in lesions of leprosy and leishmaniasis patients (260,261). Increased frequencies of  $\gamma/\delta$  T cells in PBMC from mucosal and visceral leishmaniasis patients have also been observed. Furthermore, T cell lines generated by stimulating PBMC with promastigote lysates of either *L. amazonensis* or *L. braziliensis* contain 25% to 60%  $\gamma/\delta$  T cells. In this study,  $\gamma/\delta$  T cells from a patient with mucocutaneous leishmaniasis was observed to proliferate in response to *L. donovani* HSP70 (262). In a murine model of *L. major* infection,  $\gamma/\delta$  T cells expand in both susceptible Balb/c and resistant CBA/J mice. However, while in CBA/J mice these cells return to normal levels after resolution of lesions, in chronically infected Balb/c the frequency of these cells increases up to 35% of CD3<sup>+</sup> cells in the spleen. Furthermore, treatment of susceptible and resistant mice with a mAb against  $\gamma/\delta$  TCR results in exacerbation of disease in both strains (263). These studies indicate that  $\gamma/\delta$  T cells are involved in the host response to *Leishmania*. The precise role of these cells in the immune response to leishmania HSPs requires further study.



### 3. Summary

*Leishmania* undergo abrupt changes to a higher temperature, lower pH and exposure to toxic metabolites and enzymes during transmission from the insect vector to the mammalian host. This dramatic adaptation may require the presence of leishmania HSPs. This view is supported by experiments indicating that exposure of leishmania promastigotes to 37<sup>0</sup> C results in (i) expression of several HSP families, (ii) transformation of parasites into metacyclic infective populations, (iii) increased infectivity for both macrophages in vitro and animals in vivo (iv) enhanced resistance to ROI, and (v) transformation of promastigotes into amastigotes when in addition to high temperatures, low pH is also present.

Most leishmania HSP families have been found to be expressed upon exposure of promastigotes to high temperatures. Both HSP70 and HSP83 are abundant proteins in leishmania growing at 27<sup>0</sup> C and are increased upon heat shock at 37<sup>0</sup> C. In addition, both leishmania HSP70 and HSP83 are recognized by antibodies and T cells from patients infected with *Leishmania*. Antibodies against leishmania HSP70 are found in 50% of VL patients and in sera from patients with MCL. In both diseases infected sera, (i) recognize non-conserved regions (carboxy-terminus) of the molecule, and (ii) do not cross-react with HSP70s isolated from the organisms causing Chagas' disease, malaria, schistosomiasis, and leprosy. Furthermore, leishmania HSP83 and HSP70 are recognized by T cells of patients with either CL or MCL, and leishmania HSP70 is also recognized by T cells from asymptomatic VL patients. Since most of these T cells secrete IFN- $\gamma$  and IL-2 upon stimulation in vitro with these leishmania HSPs, it is believed that those HSPs may be involved in protection rather than disease promotion. While  $\alpha/\beta$  T cell responses appear to be dominant features in resistance to leishmania infection, the role played by  $\gamma/\delta$  T cells is less clear.  $\gamma/\delta$  T cells have been found to be expanded in both murine and human leishmaniasis and at least some subsets of  $\gamma/\delta$  T cells appear to respond to leishmania HSP70.

## E. RATIONALE AND HYPOTHESES

During invasion of host cells, intracellular pathogens are exposed to conditions characterized by elevated temperature, low pH, nutrient deprivation, proteolytic enzymes, the presence of nitric oxide (NO) and other ROI. These conditions have toxic potential not only for invading pathogens, but also for the host cell. Research has shown that resistance to stressful conditions -by both host and microbe- may involve the action of HSPs. For example, phagocytosis of red blood cells by macrophages has been found to induce expression of host HSPs including principally HSP70 and heme oxygenase. Viral infection or treatment with IFN- $\gamma$  induces macrophages to express HSP60 and by virtue of cross-reactive determinants with mycobacterial HSP65, these cells are recognized by cytotoxic T cells generated against mycobacterial HSP65. On the other hand, *S. typhimurium* overexpresses both GroEL and DnaK during infection of macrophages and these HSPs are essential for survival of the pathogen within the infected cell. Exposure of leishmania promastigotes to either heat shock or to sublethal concentrations of H<sub>2</sub>O<sub>2</sub> induces expression of HSPs and this renders the cells more resistant to otherwise lethal concentrations of H<sub>2</sub>O<sub>2</sub>. These findings suggest the possibility that macrophages "stressed" by infection with *Leishmania* may have altered expression of cellular HSPs. Furthermore, exposure of macrophages in vivo to IFN- $\gamma$  may result in altered expression of host HSPs. Changes such as these may have implications for host defense since cells "stressed" by either leishmania infection, IFN- $\gamma$  or both may be recognized by HSP-specific T lymphocytes, cytotoxic or otherwise.

Compelling evidence also suggests that HSPs from a variety of pathogens are potent immunogens during infection in both humans and in animal models. Both humoral immunity and T cells responses to bacterial and parasitic HSPs have been identified. The mechanisms and functional roles of these immune responses to HSPs are not completely understood. T cell responses to mycobacterial HSP60 and HSP70 are found in both tuberculosis and leprosy patients and T cell responses to leishmania HSP70 have been found in patients with leishmaniasis. These findings indicate that HSPs are processed and presented by macrophages to T cells. Other evidence suggests that immune responses to HSP70s during parasitic

infections are directed against non-conserved epitopes of this molecule. Similarly, patients infected with leishmania develop leishmania HSP-specific CD4<sup>+</sup> Th1 type cells. These findings raise the possibility that immune responses to HSPs may be protective. Immunization studies have demonstrated that HSP60 from diverse intracellular pathogens can confer a high degree of protection against subsequent infection and that protection is mainly mediated by HSP60-specific T cells. Thus, these findings suggest the possibility that leishmania HSP60 may be recognized during infection and that recognition may be related to protection.

Research carried out during the course this thesis was intended to test two hypotheses: (i) the interaction of leishmania with macrophages involves modulation of heat shock protein expression, and (ii) leishmania HSP60 is recognized during infection. Recognition of leishmania HSP60 under these conditions has the potential to engender a protective immune response.

## F. THESIS OBJECTIVES

### 1. To examine the expression of HSPs by macrophages

**1.a.** Induction of HSP expression is examined in macrophages incubated with IFN- $\gamma$  and physical agents such as heat shock and sodium arsenite. Characterization of HSPs expression is carried out by metabolic labeling with [<sup>35</sup>S]-methionine and by immunoblotting with anti-HSP antibodies.

**1.b.** Induction of HSP expression in macrophages infected with *Leishmania* is examined using anti-HSP specific antibodies

### 2. To examine the immune response to leishmania HSP60

**2.a.** Cloning, sequencing and expression of the *L. major* HSP60 gene.

**2.b.** Recombinant LHSP60 (rLHSP60) is used (i) to determine whether patients with American Cutaneous Leishmaniasis develop an antibody response to LHSP60, and (ii) to assess the potential of LHSP60 as a vaccine candidate in susceptible Balb/c mice in combination with IL-12.

## VIII. MATERIALS AND METHODS

### A. MATERIALS

#### 1. Antibodies.

The following antibodies were from Stressgen (Victoria, B.C. Canada): Rabbit polyclonal antibodies raised against either *H. virescens* HSP60 (SPA 805) (264) or *Cyanobacteria* HSP60 (SPA 804) (265), mouse monoclonal antibody (mAb) (IgG1) specific for the inducible form of HSP70 from HeLa cells (SPA-810), and mouse mAb (IgG1) raised against HSP90 from the water mold *Achlya ambisexualis* (SPA-830). Mouse mAb D4F18 (IgG1) raised against a recombinant 72 kDa protein from *P. falciparum* (266) and also known to recognize both mammalian HSP70 was a kind gift from Dr. O. Bensaude, Génétique Moléculaire, Ecole Normale Supérieure, Paris, France. Mouse monoclonal antibody (IgG1) to the leishmania surface protease gp63 was kindly provided by Dr. W. R. McMaster, University of British Columbia, Vancouver, Canada. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from Bio-Rad (Richmond, CA), HRP-conjugated goat anti-human IgG and alkaline phosphatase-conjugated goat anti-human IgG were from Gibco BRL (Burlington, Ontario, Canada). HRP-conjugated goat anti-mouse IgG was from Tago Inc. (Burlingame, CA). To control for specificity of the rabbit polyclonal anti-HSP65, separate blotted membranes were probed with normal rabbit serum (Sigma, St. Louis, MO), and to control for specificity of the mouse monoclonal antibodies, MOPC 21 (IgG1) (Sigma, St. Louis, MO) was used.

#### 2. Reagents

Recombinant mycobacterial HSP65 was provided by Dr. R. van der See, National Institute of Public Health and Environmental Hygiene, Bilthoven, Netherlands. Recombinant gp63, the *L. major* EMBL-3 genomic library (267), *E. coli* LE392, BL21(DE3)pLysS and plasmid pET-3a (268) were provided by Dr. W.R.McMaster. p-Nitrophenyl phosphate (pNPP) was from Sigma (St. Louis, Mo.). Restriction Enzymes (RE) were from Boehringer

Manheim (Laval, Quebec, Canada) and vent DNA polymerase was from New England Biolabs (Mississauga, Ontario, Canada). Molecular weight markers used were Rainbow Markers (Amersham, UK).

## B. STRAINS AND MEDIA

### 1. Leishmania

The Sudan strain 2S of *L. donovani* or the NIH strain Friedlin of *L. major* were maintained by serial passage respectively in Syrian hamsters and mice. For experiments, hamster spleens were processed as described (269) and promastigotes were obtained by culturing amastigotes in Senekjiei's medium (270) for 10 days (stationary phase) at room temperature. Promastigotes were harvested by centrifugation at  $1,200 \times g$  for 10 min, washed 3 times in Hanks balanced salt solution (HBSS) and resuspended at  $1.6 \times 10^7$  organisms/ml in RPMI containing 10% fetal calf serum (FCS), penicillin (P) 100 U/ml, and streptomycin (S) 100 µg/ml.

### 2. Bacteria

*E. coli* (*E. coli*) strain LE392 was used as a host strain for screening the *L. major* genomic library. The genotype of strain LE392 is  $F^-$ ,  $hsdR514(r_k^-, m_k^-)$ ,  $supE44$ ,  $supF58$ ,  $\Delta$  (lacIZY)6,  $galK2$ ,  $galT22$ ,  $metB1$ ,  $trpR55$ ,  $\lambda^-$ . *E. coli* strain BL21(DE3)pLysS was used to subclone and express the leishmania *HSP60* gene. This B strain BL21 ( $F^-$ ,  $ompT$ ,  $r_B^- m_B^-$ ) is deficient in *lon* proteases and it also lacks the *ompT* outer membrane protease that can degrade proteins during purification (271). *E. coli* strain XL1-Blue was used for subcloning fragments for sequencing. This strain is Tn10,  $lacIq$ , and  $lac\Delta M15$  on the  $F'$  episome which allows screening of colonies containing inserts on the basis of  $\alpha$ -complementation. Strains of *E. coli* were grown in LBMg medium (272) at  $37^\circ C$  with aeration and were maintained on LB plates (LB medium and 1.5% agar). *Yersinia pseudotuberculosis* strain YP IIIP<sup>+</sup> was provided by Dr. B. Finlay, Department of Microbiology, University of British Columbia. Bacteria were grown in Luria broth at  $30^\circ C$  with moderate agitation (250 cycles/min) in a rotary shaker.

### C. STANDARD TECHNIQUES

Standard molecular biology techniques were carried out according to protocols described by Maniatis (272). Unless otherwise indicated the following procedures were used. Mini-preparations of plasmid DNA were done using alkaline lysis. EMBL-3 phage DNA preparations were carried out by the plate lysis procedure. Phenol extractions of plasmid DNA involved the addition of phenol/chloroform/isoamylalcohol (25/24/1, v/v/v) to an equal volume of DNA sample. Ethanol (EtOH) precipitations were performed by the addition of a one tenth volume of 2.5 M sodium acetate (NaAc) and two volumes of ice-cold 95% EtOH to the DNA solution. After incubation at  $-20^{\circ}\text{C}$  for, 10 min, the DNA was collected by microcentrifugation at 15,000 g. Precipitated DNA was washed once with 70% EtOH, lyophilized and resuspended in the appropriate solution. Agarose gels were prepared using between 0.5% to 1% agarose and 1  $\mu\text{g}/\text{ml}$  ethidium bromide (EtBr) in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.0). DNA samples were loaded for electrophoresis in one tenth volume of sample buffer containing 10% glycerol, 0.04% bromophenol Blue in 1 x TAE buffer.

### D. PREPARATION OF BONE MARROW-DERIVED MACROPHAGES (BMM)

Cells were flushed from the tibias and femurs of four, 6-8 week old female C57BL/6 mice using a 21 gauge needle attached to a 10 ml syringe containing 10 ml of RPMI medium as described (273). After centrifugation at  $800 \times g$  for 10 min, cells were incubated at  $4^{\circ}\text{C}$  in 5-10 ml of Tris- $\text{NH}_4\text{Cl}$  (9 volumes of 0.83 %  $\text{NH}_4\text{Cl}$  to 1 volume of 0.17 M tris, pH 7.2) to lyse contaminating erythrocytes (269). Cells were then washed once in RPMI and debris and non-dispersed tissue removed by filtering the suspension through a 10 ml disposable pipette fitted with a piece of sterile cotton. Cells were then centrifuged, resuspended in 16 ml of RPMI supplemented with 10% FCS, P/S and 10% conditioned medium obtained from EMT-6 cells as a source of CSF-1 as described (274) and dispensed into a single 100 mm x 15 mm cell culture dish (Fisher Scientific Co., Canada). After incubation for 24 hr at  $37^{\circ}\text{C}$  in a 95%

air/5% CO<sub>2</sub> humidified atmosphere, non-adherent cells (undifferentiated bone marrow cells) were collected in a 50 ml tube, and the cell suspension was brought to a final volume of 48 ml, including 10% EMT-6 conditioned medium. The suspension was divided in equal portions and dispensed into 4 disposable 75 cm<sup>2</sup> cell culture flasks (Corning, N.Y.) and incubated at 37° C in 95% air/5% CO<sub>2</sub> for 7 days with the addition of fresh 10% EMT-6 conditioned medium on day 3. At the end of the incubation period (day 8) homogeneous monolayers of approximately 10<sup>7</sup> adherent cells per flask were obtained. The monolayers were washed gently with warm RPMI followed by further incubation for 24 hr in fresh RPMI minus EMT-6 conditioned medium. On day 9, phagocytic activity was assessed and found to be > 98 % when cells were incubated for 24 hr with a 0.002 % (w/v) suspension of fixed *S. aureus* as described (275).

#### E. INFECTION OF MACROPHAGES

Monolayers were infected with *Leishmania* by adding 12 ml of a suspension of stationary phase promastigotes at  $1.6 \times 10^7$  parasites/ml to each flask. After 3 hr of incubation at 37° C, non-ingested organisms were removed by washing flasks three times with warm HBSS. To monitor infection, Diff-Quik-stained cytocentrifuge preparations were routinely prepared at this point using cells dislodged from monolayers. These analyses showed that no extracellular promastigotes were present and that all intracellular forms had the morphology of amastigotes. For experiments, cells were then either harvested immediately and processed for Western blotting or were allowed to incubate in 12 ml of fresh medium to complete 24 or 48 hr of culture. For infection with *Y. pseudotuberculosis*, bacteria were grown overnight in Luria broth at 30° C with moderate agitation (250 cycles/min) in a rotary shaker. The concentration of bacteria was adjusted by OD<sub>600</sub>, which corresponded to a concentration of  $2 = 1.6 \times 10^9$  organisms /ml. *Yersinia* were washed 3 times in warm HBSS and resuspended at a concentration of  $0.8 \times 10^8$  per ml in RPMI with 10% FCS and P/S and were used to infect macrophage monolayers at an approximate ratio of 100 bacteria/cell.

## F. SODIUM ARSENITE AND HEAT SHOCK TREATMENTS

Macrophage monolayers were washed with warm RPMI and incubated in the same medium containing 10 % FCS, P/S and NaAsO<sub>2</sub> (40 uM) (Fisher Scientific, Vancouver, BC, Canada). After 3 hr of incubation, cells were washed and then incubated for 1 hr in RPMI containing 10 % FCS and P/S, but without NaAsO<sub>2</sub>. Control cells were processed in parallel, but without addition of NaAsO<sub>2</sub>. NaAsO<sub>2</sub>-treated and control cells were harvested and prepared for Western blotting. For heat shock treatment, macrophage monolayers were gently washed in warm RPMI, replenished with fresh RPMI supplemented with 10% FCS and P/S and incubated at 42° C for 20 min. After incubation, cells were allowed to recover at 37° for 1 hr and then processed for Western blotting. Control cells were prepared in parallel, but were incubated at 37° C instead of 42° C.

## G. METABOLIC LABELING

BMM were either untreated or infected either with *Leishmania* or *Yersinia* or treated with NaAsO<sub>2</sub> as indicated above and then incubated for appropriate periods as indicated in figure legends. The culture medium was then removed and the cells washed with RPMI without methionine and then labeled for 45 min with [<sup>35</sup>S]methionine at 100 µCi/ml (1,020 Ci/mmol; 1 Ci = 37 Gbq, Amersham). After labeling, the medium was removed and the cells washed with cold HBSS. Cells were dislodged with a cell scraper (Costar Corporation, Cambridge, MA), counted and solubilized by adding SDS sample buffer. Samples were then boiled for 5 min and loaded on to SDS-PAGE gels. Following electrophoresis, gels were stained with Coomassie blue, dried and autoradiographed with Kodak X-Omat films.

## H. IMMUNOBLOTTING

After the indicated treatments were completed, monolayers of BMM were placed on ice for 10 min and were then dislodged with a cell scraper in 10 ml of HBSS and centrifuged at



800 x g for 10 min. Cells were then resuspended in HBSS and washed three times by microcentrifugation (Microcentrifuge E, Beckman) for 30 sec. Before the final wash, sample aliquots were taken to determine cell numbers, intensity of infection (Diff Quik Staining, American Scientific Products) and also for determination of protein concentration (as described below). Cells were then suspended in Laemmli sample buffer (276) at a concentration of  $2 \times 10^7$  cells/ml, boiled at 95-100° C for 5 min. used and either immediately or stored at -20° C. For immunoblotting, 60 ug of cell protein was separated on 10% SDS-PAGE slab gels and transferred to nitocellulose membranes (Bio Rad, Richmond, CA.) using a Multiphor II electrophoresis unit (LKB, Bromma, Sweden). After transfer, membranes were washed briefly in PBS with 0.1% (w/v) Tween and then processed according to the manufacturer's instructions for enhanced chemiluminescence (ECL) (Amersham, UK). Briefly, membranes were blocked for non-specific binding with 5% skim milk (Difco Laboratories, Detroit) for 1 hr and then probed with the indicated primary antibody for 1 hr. After extensive washing, an appropriate secondary antibody was added for 40 min, followed by washing and development of the ECL signal using ECL film according to the manufacturer's instructions.

## I. PROTEIN MEASUREMENTS

Determination of protein content of samples were done using the DC Protein Assay System (Bio Rad, Richmond, CA) for measurement of protein content in the presence of detergent, using an ELISA plate reader (Titertek Multiscan MC, Helsinki, Finland) at a wavelength of 690 nm.

## J. MOLECULAR CLONING OF HSP60

Cloning of HSP60 from *L. major* was carried out using a DNA probe produced by polymerase chain reaction (PCR). A segment of DNA flanked by nucleotide sequences encoding two highly conserved regions (GDGTTATV and AVKAPGFGD) in the HSP60 family of proteins was amplified by PCR from genomic DNA of *L. major* (NIH Friedlin strain) using the degenerate oligonucleotide primers; 5'-

GGNGAYGGNACNACNACNGCNACNGT-3' and 5'-TCNCCRAANCCNGGNGCYTTNACNGC-3', where N = A, C, G or T, R = A or G and Y = C or T. The conditions used for PCR were the same as described earlier (268,277). PCR amplification carried out using the above set of oligonucleotide primers and *L. major* genomic DNA, resulted in amplification of a fragment of approximately 0.6 Kb as expected based on the position of primers in the *HSP60* sequence (268) (data not shown). Comparison of the derived sequence with sequences in various databases (278) indicated that the sequenced PCR fragment from *L. major* was unique, but that it showed highly significant and extensive similarity to *HSP60* sequences from various species. The DNA of the cloned PCR fragment was used as a probe in subsequent studies to screen the *L. major*  $\lambda$ EMBL-3 genomic DNA library for isolation of the *HSP60* gene.

One-half ml of an overnight culture of *E. coli* (LE392) was infected at 37° C for 20 min with 30,000 pfu from the  $\lambda$ EMBL-3 *L. major* genomic DNA library. The suspension was mixed with top agarose, plated on 150 mm plates (Fisher Scientific, Vancouver, BC, Canada) containing agar and incubated for 12 hr at 37° C. Plaques were transferred to nylon membranes (Boehringer Mannheim, Laval, Quebec, Canada) and plaque hybridization was carried out with the PCR-generated *L. major* *HSP60* probe. Labeling of the probe and hybridization at high stringency conditions was carried out using ECL (Boehringer Mannheim, Laval, Quebec, Canada).

## K. DNA PREPARATION

High molecular weight genomic DNA was isolated from *L. major* promastigotes as described (279). *Leishmania* were grown to early log phase and then pelleted at 10,000 x g for 5 min at 4°C. Cells were washed in PBS (140 mM NaCl, 3 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>) once prior to lysis in a pre-heated solution containing 0.5 M EDTA, 0.5% N-lauryl sarcosine and 100 µg/ml Proteinase K. After incubation at 50°C overnight, DNA was extracted twice with phenol followed by chloroform and dialyzed extensively against TE buffer (10 mM Tris, 1mM EDTA pH 7.5). Contaminating RNA was removed by incubating

with heat-treated RNase A to a final concentration of 100 µg/ml and incubation at 37°C for 1 hr. DNA isolation from λEMBL-3 clone 232, was carried out using a Nucleobond AX kit (Macherey-Nagel, Germany). Plasmid DNA isolation was carried out using either a standard protocol of phenol-chloroform extraction (272) or a QIAGEN-tip 20 DNA extraction kit (Qiagen, Chatsworth, CA). Preparation of DNA for nested deletions was carried out by cesium chloride gradient centrifugation (272) .

#### **L. RESTRICTION ENZYME ANALYSIS AND SOUTHERN BLOTTING**

Clone 232 from the λEMBL-3 *L. major* DNA library, which hybridized strongly with the PCR-generated, HSP60 DNA probe was characterized by restriction enzyme (RE) and Southern analysis using standard conditions. One µg of either genomic or λEMBL-3 DNA was digested with 20 units of the desired restriction enzyme at 37°C for 3 hr. After digestion, DNA fragments were separated in 1% agarose gels and transferred by capillary action to nylon membranes in 0.4M NaOH. The HSP60 DNA probe was labeled by the random priming method (280). Nucleotides dATP, dCTP and dGTP (1 mM each) were mixed with 0.65 mM dTTP and 0.35 mM dUTP-digoxigenin (Boehringer Mannheim, Laval, Quebec, Canada). This mixture was then combined with random oligonucleotide primers and with 50 ng of template DNA. The elongation reaction was carried out in the presence of 2U Klenow reagent for 1 hr at 37°C followed by 19 hr at rt. The labeled probe was separated in 1% low-melting-point (LMP) agarose and purified from the gel using a QIAquick gel extraction kit (Qiagen, Chatsworth, CA). Southern blotting was carried out under high stringency conditions and blots were developed by ECL.

#### **M. DNA SEQUENCING**

The *Sal* I DNA fragment isolated from clone 232 was subcloned into the *Sal* I site of the pGEM-3Z f (+) sequencing vector (Promega, Madison, WI) and two subclones (clone 25 and 27) of this construct, representing opposite orientations of the insert, were isolated for sequencing. A series of overlapping nested deletion subclones were generated for each clone.

Forty subclones containing overlapping fragment sizes in the range of 100-250 bp were sequenced by the dideoxy, chain termination method using an automated sequencer (Applied Biosystems). Sequence data was analyzed using the Geneworks analysis program (Intelligenetics, Mountain View, CA). Both strands were sequenced resulting in an unambiguous consensus sequence.

## N. SEQUENCE COMPARISON AND ANALYSIS

Sequences of *HSP60* homologs from other species were obtained from Genbank and Swissprot databases. The sources and accession numbers of these sequences are: human (P10809, sp), mouse, (X53584, gb), rat (S11159, gb), *H. virescens* (P25420, sp), hamster (P18687, sp), *Saccharomyces cerevisiae* (P19882, sp), maize (P29185, sp), *Arabidopsis thaliana* (P29197, sp), *Cucurbita* (X70868, gb), *Brassica napus* (Z27165, gb), *P. falciparum* (X75420, gp), *Trypanosoma cruzi* (L0879, gb), *E. coli* (P05380, sp) and *Mycobacterium leprae* (P09239, sp). Pairwise similarity between protein sequences was determined using the PALIGN program from the PC Gene Software using structure gene matrix and unit gap and open gap costs respectively of 2 and 7. Multiple alignment of sequences was carried out using the CLUSTAL program from the PC Gene package. Any misalignments detected by visual inspection were corrected manually. Evolutionary relationships based on *HSP60* sequences were determined using the programs BOOT, NEIGHBOR and CONSENSE from PHYLIP version 3.5 program package (281).

## O. EXPRESSION OF RECOMBINANT LEISHMANIA HSP60 (rLHSP60)

The *LHSP60* gene was amplified by PCR using forward and reverse primers designed from the initiation and termination sequences of the gene (Figure 4). In order to subclone the PCR product into the *Nde* I site of the pET-3a expression vector, *Nde* I linkers were included in both primers, one at the ATG start codon and a second at the end of the TGA termination codon. *Nde* I site was chosen because it allows inclusion in frame of the ATG start codon immediately after the promoter. PCR reactions were carried out in a Thermal Cycler 480

(Perkin Elmer Cetus, Norwalk, CT) using Vent DNA Polymerase (New England Biolabs, Mississauga, Ontario, Canada) under conditions of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and primer extension at 72°C for 10 min. The PCR product was purified from LMP agarose gel (Gibco BRL, Burlington, Ontario, Canada) using a QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The purified product was digested with *Nde* I and subcloned into the pET-3a plasmid under the T7 RNA polymerase promoter using standard techniques (272). pET-3a allows the gene to be expressed right after the T7 RNA polymerase promoter. Thus, the recombinant protein, which does not contain a fusion protein, localizes in inclusion bodies from which it can be easily purified (271,282). Plasmids carrying the gene in the proper orientation were used to transform *E. coli* BL21(DE3)pLysS (271,282). Expression of the *HSP60* gene in *E. coli* was carried out by inducing the *lac* promoter for the expression of T7 RNA polymerase using (IPTG).

#### **P. PURIFICATION OF (rLHSP60)**

Large scale purification of rLHSP60 was carried out as described (282). Briefly, 250 ml of LB medium containing ampicillin (100 ug/ml) and chloramphenicol (25 ug/ml) was inoculated with 2.5 ml of an overnight culture of clone pET-LHSP60 expressing the recombinant protein. The culture was incubated on a rotary shaker at 37°C until log phase growth ( $A_{600} = 0.8$ ) followed by induction of the T7 RNA polymerase with 0.2 mM IPTG and incubation for an additional 3 hr. Bacteria were pelleted by centrifugation at 4°C and lysed overnight at -20°C in 5 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl and 1 mM EDTA. Inclusion bodies (IB) containing insoluble recombinant protein were collected by centrifugation at 8,000 rpm for 10 min at 4°C and washed extensively with buffer containing 20 mM Tris-HCl, 20 mM NaCl and 1 mM EDTA. Recombinant protein was purified from IBs by 2 rounds of separation on and elution by diffusion from preparative SDS-PAGE gels. Purified protein was concentrated by ultrafiltration with Centriprep-30 (Amicon), dialyzed extensively against TBS buffer and filter sterilized with 0.22  $\mu$ M filters (Millipore).

## **Q. TWO-DIMENSIONAL GEL ELECTROPHORESIS**

rLHSP60 was analyzed further by 2-D gel electrophoresis (283). Briefly, 0.5  $\mu$ g of purified recombinant protein was diluted in IEF sample buffer containing 10% (v/v)  $\beta$ -mercaptoethanol, 20% (v/v) glycerol, 9 M urea, 3% (v/v) NP-40, 0.8% (v/v) ampholines pH 5 to 8, and 0.2 % (v/v) ampholines pH 3 to 10. IEF was carried out in gel rods (10 cm and 2.5 mm) with 9 M urea, 2% NP-40 (v/v), 30% acrylamide, 0.8% NN-bis-methylene-acrylamide, 4% (v/v) ampholines pH 5 to 8 and 1% (v/v) ampholines pH 3 to 10. Samples were focused at 100 V for 1 hr, 200 V for 2 hr, 400 V for 16 hr and 800 V for 2 hr. The upper chamber (cathode) contained 25 mM NaOH and the lower chamber (anode) 0.2% H<sub>3</sub>PO<sub>4</sub>. After focusing, gels were equilibrated in SDS-PAGE sample buffer and run in a second dimension of SDS-PAGE (10%).

## **R. PATIENT SERA**

Sera from patients with American Cutaneous Leishmaniasis were obtained from the serum bank of the Centro Internacional de Investigaciones Medicas (CIDEIM), Cali, Colombia. These patients were referred to CIDEIM for diagnosis and treatment. All patients were diagnosed with cutaneous leishmaniasis by isolation of leishmania from lesions as described elsewhere (284). The following sera were tested (numbers refer to codes assigned to patients at CIDEIM): 4319, 4124, JE, 2049, 4245, 4104, 4676, 4282, 4289, 4322, JO, ZL, 4289, 4013, 1007. Sera from five healthy donors living in Vancouver and who had never visited a leishmania endemic area were used as negative controls. These are designated: 94512, 94527, 94826, 948262 and 92624.

## **S. MEASUREMENT OF SERUM ANTIBODIES TO rLHSP60**

Detection of anti-HSP60 antibodies in human immune sera was done by ELISA as described previously (285). Flat-bottomed (96-well), polystyrene, microtiter plates (Falcon) were coated with either rLHSP60, recombinant mycobacterial HSP65 (rMHSP65) (5 $\mu$ g/ml, 100  $\mu$ l/well) or with coating buffer alone. Plates were incubated at room temperature for 24

hr and then washed three times with PBS-Tween. Non-specific binding sites were blocked by adding 3% BSA in PBS-Tween to all wells for 1 hr at 37°C. After three washings, either test or control sera were added at 1:4000 dilution in PBS/BSA-Tween and incubated for 1 hr at 37°C. Plates were then washed again prior to addition of a 1:4000 dilution of alkaline phosphatase-conjugated goat anti-human secondary antibody in PBS/BSA-Tween for 1 hr at 37°C. After 5 washings, wells were incubated with pNPP at a concentration of 1 mg/ml in substrate buffer (0.5 mM MgCl<sub>2</sub> in 1 M diethanolamine, pH 9.8) for 15 min at 37°C. Measurements of optical density were carried out using a Dynateck MR5000 ELISA plate reader (Chantilly, VA) at a wavelength 405 nm. To control for non-specific binding of serum components, the absorbance of antigen-free wells was subtracted from that of the antigen-coated wells to obtain a value for specific absorbance.

#### **T. IMMUNIZATION OF MICE**

Balb/c mice were immunized as described (213). Groups of mice (5 per group) were immunized intradermal in the right footpad with either IL-12 plus (1 µg), rLHSP60 (25 µg) or a combination of IL-12/rLHSP60 (1 and 25 µg respectively) in a total volume of 50 µl TBS. An additional group received 50 µl of TBS and another group received no treatment. Ten days later mice received a second immunization in the flank, this time using 12.5 µg of rLHSP60 and 1 µg IL-12. Two weeks later mice were immunized on the flank again and then infected in the left footpad with  $2 \times 10^6$  stationary phase promastigotes of *L. major*. The course of disease was monitored by measuring the thickness of infected footpads at weekly intervals using a caliper. Contralateral uninfected footpads were also measured and used as controls. At the end of seven weeks, mice were sacrificed, parasite loads in infected tissue were determined and spleen and popliteal lymph node cells tested in a proliferation assay using the rLHSP60 as antigen.

## U. QUANTITATION OF PARASITES IN INFECTED TISSUE

Parasites in infected tissue (footpad) were enumerated by LDA assay as described previously (286) using liquid culture medium (LCM) (287). Footpads were removed between the heel and the toes and then weighed. Tissue was cut into pieces in LCM and homogenized on ice until completely disrupted using a glass tissue homogenizer. Homogenates were allowed to settle for 1 min to remove large debris and then serial ten-fold dilutions of supernatants were made in LCM. Aliquots of 0.1 ml of each serial dilutions were distributed into 12 replicate wells of 96-well flat-bottom plates (Falcon). These were placed in plastic bags containing a wetted paper towel and incubated at 26° C for 10 days. Parasite growth was determined by the presence of motile promastigotes in wells using an inverted microscope. Parasite frequencies in infected tissue were analyzed using a computer program originally designed for the determination of frequencies of antigen-specific T-cells in test populations of cells subjected to limiting dilution assay (286,288).

## V. PROLIFERATION OF SPLEEN AND LYMPH NODE CELLS

Spleen and popliteal lymph node cells were prepared as described previously (289,290). Cells were flushed from spleens with 5 ml HBSS in a 10 ml syringe and 22 G needle. Single cell suspensions were obtained by gentle homogenization. Cells were treated with Tris-buffered ammonium chloride to lyse red blood cells (269) and then washed three times in RPMI 1640. Popliteal lymph nodes were prepared by passage through stainless steel mesh screens to release the cells followed by washing three times with RPMI 1640. After the final wash, spleen and popliteal lymph node cells were counted and adjusted to  $2 \times 10^6$  ml in tissue culture medium consisting of RPMI 1640, 10% heat inactivated fetal calf serum (FCS), L-glutamine (2mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol (50 µM). Cells were then dispensed into 96-well flat-bottom plates (Falcon) in 0.2-ml volumes. Concanavalin A (ConA) and rLHSP60 were added to final concentrations of respectively 5 µg/ml and 10 µg/ml. The plates were kept in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 95% air) at 37° C for 72 hr. Cells were then pulsed with [<sup>3</sup>H]thymidine (1 µCi/well), incubated for an additional 18



hr and then harvested in an automated cell harvester. Radioactivity was counted in a  $\beta$ -counter (Beckman).

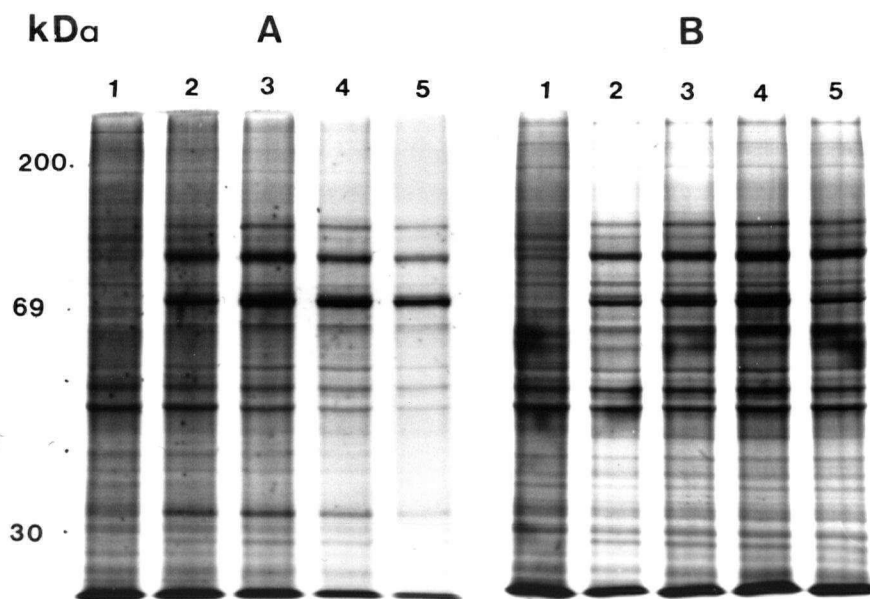
## W. STATISTICAL ANALYSIS

Data were analysed for statistical significance by using the software program "Graph Pad INSTAT, Instat Biostatistics". This includes One-way Analysis of variance (ANOVA), unpaired and paired T tests and the Tukey-Kramer Multiple Comparisons Test. Data are considered significant at  $P < 0.05$ .

## IX. RESULTS AND DISCUSSION

### A. EXPRESSION OF STRESS PROTEINS BY MACROPHAGES

Initial experiments examined the expression of heat shock proteins (HSPs) by stressed macrophages. The hypothesis being tested was that infection of macrophages with *Leishmania* or exposure of macrophages to activating cytokines such as IFN- $\gamma$  would result in increased synthesis of HSPs and perhaps, increased cell-surface expression of these proteins.

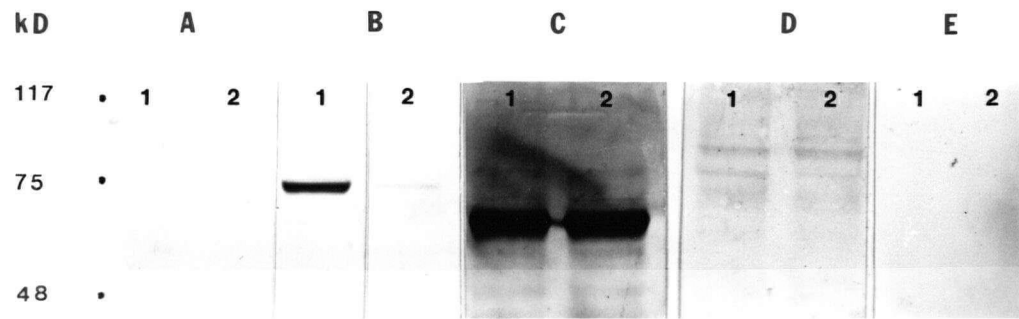


**Figure 1.** Autoradiogram of metabolic labeling of Mono-Mac-6 cells exposed to NaAsO<sub>2</sub> (A) or to heat shock (B).

Cells were either untreated (lane 1, panels A and B), treated with either 20, 40, 60 or 80  $\mu$ M of NaAsO<sub>2</sub> (panel A, lanes 2, 3, 4 and 5, respectively) or subjected to heat shock at 42<sup>o</sup> C for either 120, 60, 40 or 20 min (panel B, lanes 2, 3, 4 and 5, respectively) in methionine free medium. Cells were then pulsed with Translabel [<sup>35</sup>S]-methionine for 1.5 hr, solubilized in SDS sample buffer and subjected to 10% SDS-PAGE and autoradiography. Molecular weight markers are shown in the margin.

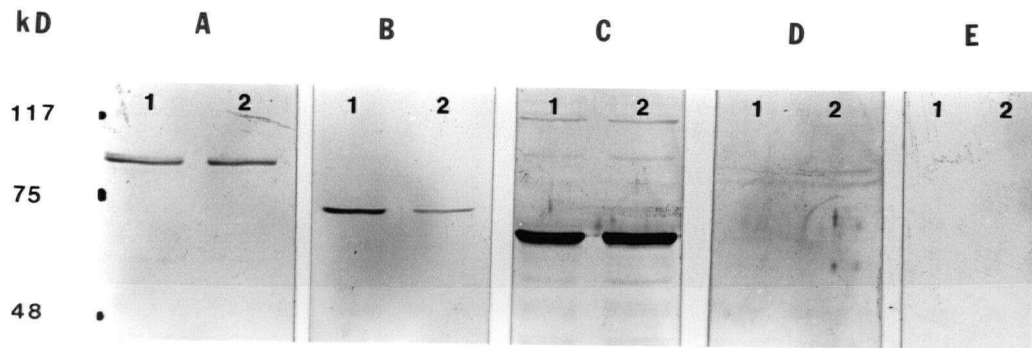
## 1. Expression of stress proteins by Mono-Mac-6 cells

An *in vitro* system consisting of metabolic labeling with [ $^{35}\text{S}$ ]-methionine and immunoblotting was established to detect expression of HSPs belonging to the 90-kDa, 70-kDa and 60-kDa families. Initial experiments involved the human macrophage cell line Mono-Mac-6 which possesses characteristics of mature monocytes. Mono-Mac-6 cells were originally isolated from the peripheral blood of a patient with monoblastic leukemia (287a). In response to increasing concentrations of NaAsO<sub>2</sub> (Figure 1A) or to HS at 42<sup>o</sup> C (Figure 1B), MonoMac-6 cells showed increased [ $^{35}\text{S}$ ]-methionine labeling of a subset of proteins of M<sub>r</sub> 110,000, 90,000, 72,000 and 65,000 while the synthesis of other proteins decreased. Of interest, a low molecular weight HSP of M<sub>r</sub> 32,000 was also induced by sodium arsenite treatment (Figure 1A). Analysis of these cell extracts by immunoblotting indicated that exposure to HS (Figure 2) or to NaAsO<sub>2</sub> (Figure 3) resulted in significantly increased expression of HSP70 (Figures 2B and 3B), with only minor, insignificant increases in either HSP60 (Figures 2C and 3C) or HSP90 (Figures 2A and 3A). Additional experiments examined whether stimulation with IFN- $\gamma$  would bring about enhancement of HSPs. In response to IFN- $\gamma$ , induction of HSPs in Mono-Mac-6 cells was not observed as determined by either labeling with [ $^{35}\text{S}$ ]-methionine or Western blotting (not shown).



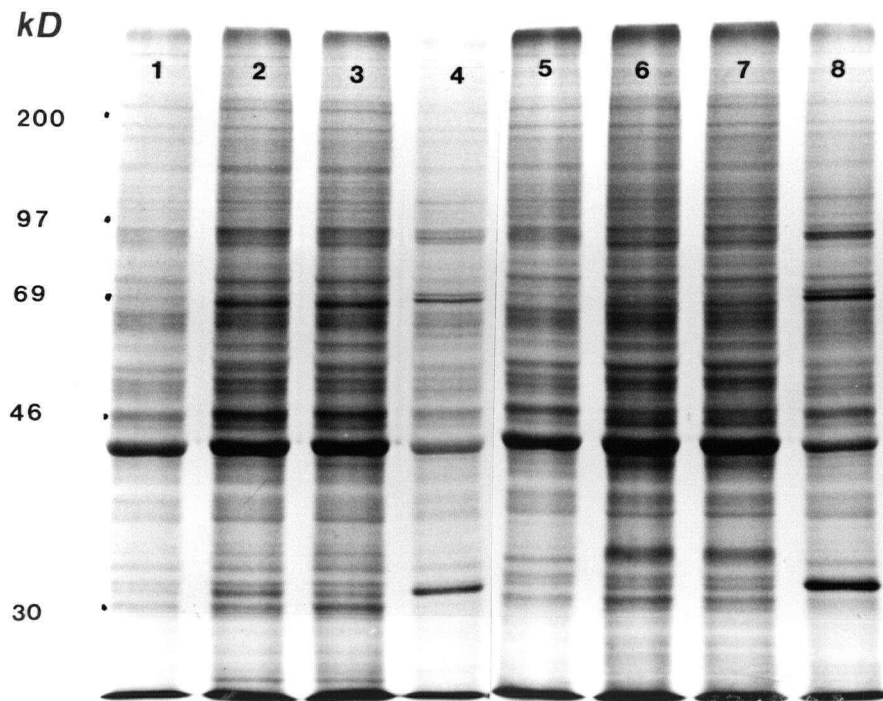
**Figure 2. Anti-HSP immunoblots of cells of the human macrophage cell line Mono-Mac-6.**

Cells were either untreated (lanes 2) or exposed to heat shock (lanes 1) at 43<sup>0</sup> C for 40 min. Cells were then returned to 37<sup>0</sup> C and 1 hr later were washed and processed in SDS sample buffer for 10% SDS-PAGE and immunoblotting using standards techniques. Molecular weight markers are shown in the margin. Panels A, anti-HSP90, B, anti-HSP70, C, anti-HSP60, D, normal rabbit serum and E, irrelevant mAB (IgG1 isotype).



**Figure 3. Anti-HSP immunoblots of Mono-Mac-6 cells exposed to NaAsO<sub>2</sub>.**

Cells were either untreated (lanes 2) or exposed to NaAsO<sub>2</sub> (lanes 1) 40  $\mu$ M for 3 hr. The remainder of the legend is as for Figure 2. Panels A, anti-HSP90, B, anti-HSP70, C, anti-HSP60, D, normal rabbit serum, and E, irrelevant IgG1 mAB.



**Figure 4. Autoradiogram of metabolic labeling of murine BMM exposed to NaAsO<sub>2</sub> or IFN- $\gamma$ .**

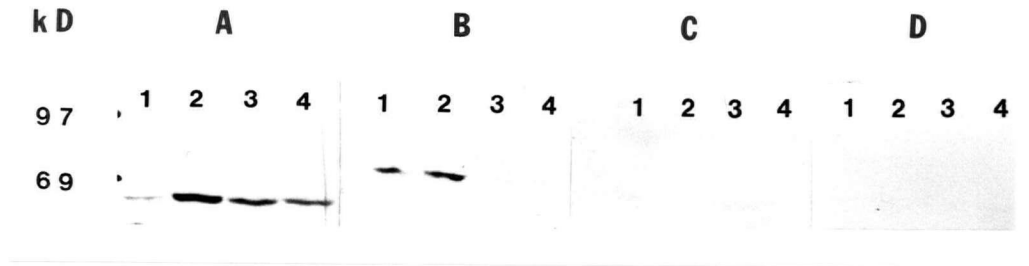
Cells were either untreated (lanes 1 and 5), treated with IFN- $\gamma$ , 1000 U/ml for either 6 hr (lane 2), 12 hr (lane 3) or 48 hr (lane 7), or with IFN- $\gamma$ , 200 U/ml for 48 hr (lane 6) in methionine free medium. Parallel cultures were treated with 40  $\mu$ M NaAsO<sub>2</sub> for the final 3 hr of either a 12 hr (lane 4) or a 48 hr (lane 8) incubation. The remainder of the legend is as for Figure 1.

## **2. Expression of stress proteins by murine bone marrow macrophages (BMM)**

Because of the lack of induction of HSPs in MonoMac-6 cells in response to IFN- $\gamma$ , an alternative *in vitro* system was established using bone marrow macrophages (BMM) from C57Bl/6 mice. In initial experiments, adherent BMM were exposed to elevated temperatures or to a range of concentrations of NaAsO<sub>2</sub>. Incubation at 43<sup>0</sup> C or with NaAsO<sub>2</sub> (40  $\mu$ M) induced optimal responses with respect to new protein synthesis. As shown in Figure 4 (lanes 4 and 8), increased [<sup>35</sup>S]-labeling of a subset of proteins was detected in cells treated with NaAsO<sub>2</sub> while synthesis of the majority of cell proteins was decreased. Treatment with

NaAsO<sub>2</sub> resulted in increased labeling of proteins within the range of M<sub>r</sub> 90,000, 70,000 and 30,000. Incubation with IFN- $\gamma$  for either 6 or 12 hr resulted in increased [<sup>35</sup>S]-labeling of proteins in the range of M<sub>r</sub> 90,000-100,000, M<sub>r</sub> 60,000-70,000, M<sub>r</sub> 50,000 and M<sub>r</sub> 25,000-30,000 (Figure 4, lanes 2 and 3, respectively). Similar results were observed when cells were exposed to IFN- $\gamma$  (either 200 or 1000 U/ml) for 48 hr (Figure 4, lanes 6 and 7) except for a prominently induced band at ~M<sub>r</sub> 34,000 in 48 hr IFN- $\gamma$ -treated cells which was not observed in cells treated for either 6 or 12 hrs. This M<sub>r</sub> 34,000 protein, which was not induced in response to NaAsO<sub>2</sub> likely represents the  $\alpha$  chain of the MHC class II molecule.

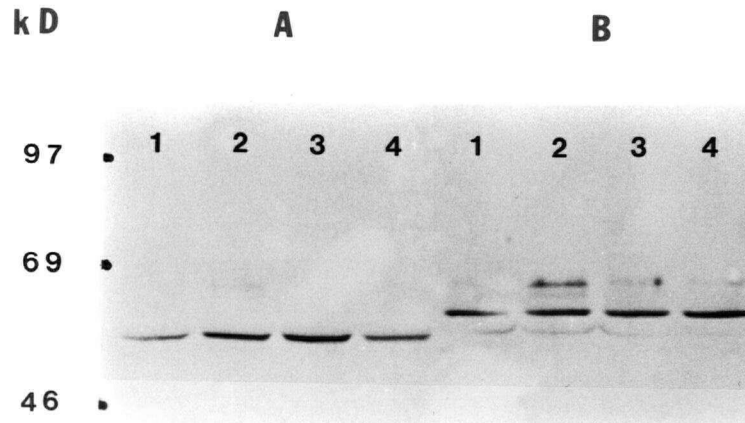
Based upon the results of [<sup>35</sup>S]-labeling experiments, total lysates of BMM treated with either NaAsO<sub>2</sub>, HS or IFN- $\gamma$  were analyzed by immunoblotting with anti-HSP antibodies. These analyses demonstrated that treatment with either NaAsO<sub>2</sub> or HS resulted in increased synthesis of HSP70 in BMM (Figure 5B). In contrast to Mono-Mac cells, however, induction of HSP90 was also observed in BMM in response to NaAsO<sub>2</sub>, modestly in response to IFN- $\gamma$  but not in response to HS (Figure 5A). An additional finding of interest in this analysis of BMM was that the antibody to HSP90 reacted consistently with a protein of M<sub>r</sub> ~65,000 and only minimally if at all with a protein in the range of M<sub>r</sub> 90,000. Since the anti-HSP90 antibody used in these experiments reacted as expected with a protein of ~M<sub>r</sub> 90,000 in Mono-Mac-6 cells (Figure 2A), it seems most likely that the NaAsO<sub>2</sub> induced protein of M<sub>r</sub> ~65,000 in BMM recognized by anti-HSP90 (Figures 5A and 6A) represents a partial degradation product of HSP90. Alternatively, monoclonal anti-HSP90 may be crossreacting with a non-HSP90 protein migrating at the 65-kDa level in murine BMM.



**Figure 5. Anti-HSP immunoblots of murine BMM.**

Cells were either untreated (lanes 4) or exposed to heat shock at 43<sup>0</sup> C for 40 min (lanes 1), NaAsO<sub>2</sub>, 40 μM x 3 hr (lanes 2) or IFN-γ, 1000 U/ml x 36 hr (lanes 3). The remainder of the legend is as for Figure 2. Molecular weight markers are shown in the margin. Panels A, anti-HSP90, B, anti-HSP70, C, normal rabbit serum, and D, irrelevant IgG1 mAb.

In contrast to HS or NaAsO<sub>2</sub>, treatment of cells with IFN-γ did not result in induction of HSP70 (Figure 5B). Exposure to IFN-γ did, however, result in a modest induction of the protein of Mr ~65 recognized by the anti-HSP90 antibody and which was also induced in response to NaAsO<sub>2</sub> (Figure 5A). The finding that HSP90 is induced in BMM in response to arsenite and IFN-γ was confirmed in additional experiments (Figure 6A). In this experiment a modest induction of HSP60 was also observed, both in response to IFN-γ and NaAsO<sub>2</sub> (Figure 6B, lower band). In a series of 6 independent experiments in which autoradiograms of immunoblots were analyzed by scanning densitometry, HSP60 expression was  $6.7 \pm 0.5$  absorbance units (mean  $\pm$  SEM) in control cells and  $8.5 \pm 0.74$  in IFN-γ treated cells ( $P=0.013$  by paired t test) and expression of HSP90 was  $6.4 \pm 0.5$  absorbance units in control and  $8.0 \pm 0.8$  in IFN-γ-treated cells ( $P=0.049$  by paired t test).



**Figure 6. Anti-HSP immunoblots of murine BMM.**

Cells were either untreated (lanes 1) or exposed to  $\text{NaAsO}_2$ , 40  $\mu\text{M}$  x 3 hr (lanes 4) or IFN- $\gamma$ , 1000 U/ml x 6 hr (lanes 2) or IFN- $\gamma$ , 1000 U/ml x 36 hr (lanes 3). The remainder of the legend is as for Figure 2. Molecular weight markers are shown in the margin. Panel A, anti-HSP90, and Panel B, anti-HSP60.

### 3. Expression of stress proteins by BMM infected with *L. donovani*

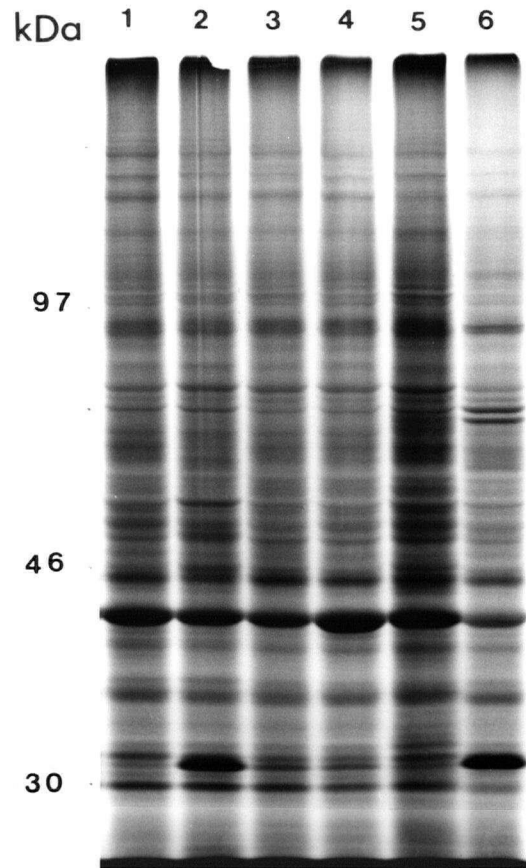
To examine HSP expression in infected cells, BMM were infected with amastigotes of *L. donovani* and incubated at 37 $^{\circ}$  C. Cells were either labeled with [ $^{35}\text{S}$ ]-methionine and extracts analyzed by SDS-PAGE or analyzed by immunoblotting using HSP-specific antibodies.

#### a) Expression of Heme Oxygenase (HO) in leishmania-infected BMM

BMM were either untreated or infected with amastigotes of *L. donovani* and incubated for either 6 or 24 hr. Infected and control cells were then labeled with [ $^{35}\text{S}$ ]-methionine. Following SDS-PAGE separation of cell extracts, analysis of autoradiographs indicated pronounced induction of a 32-kDa protein in cells that had been incubated with leishmania for 6 hr (Figure 7, lane 2). A protein of 32 kDa was also expressed in uninfected BMM treated with  $\text{NaAsO}_2$  (40  $\mu\text{M}$  x 3 hr) (Figure 7, lane 6). This protein was not observed, however, in

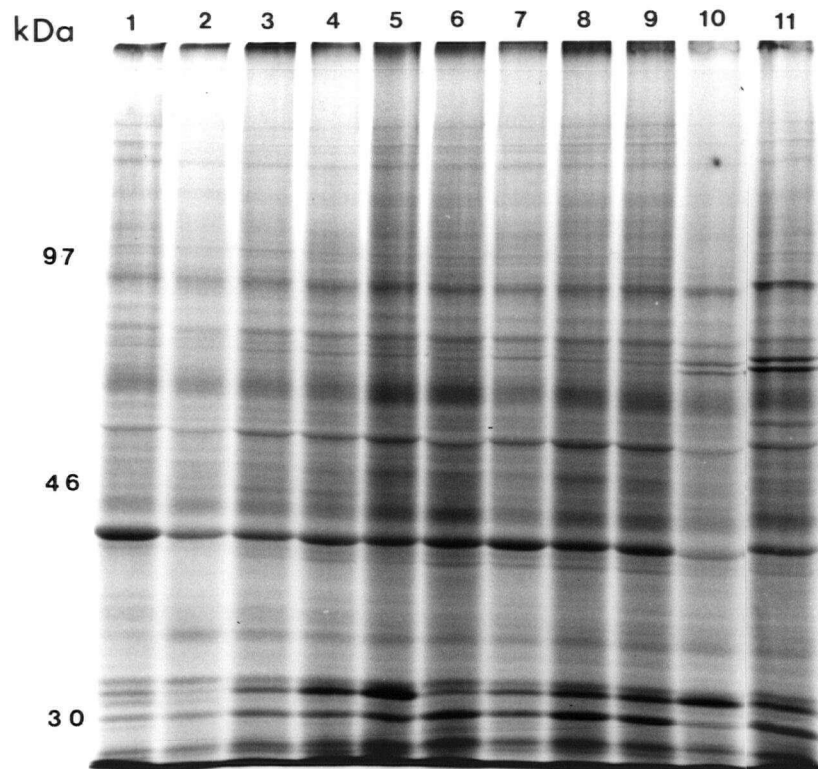


(i) cells incubated with leishmania for 24 hr (Figure 7, lane 3), (ii) uninfected BMM that had ingested fixed *S. aureus* and were incubated for 24 hr (Figure 7, lane 4), or (iii) BMM treated with IFN- $\gamma$  (1000 U/ml x 36 hr) (Figure 7, lane 5). In addition, a 32 kDa band was not observed to be induced when leishmania parasites alone were labeled in the presence of [ $^{35}$ S]-methionine (not shown). Of note, no parasite-derived bands in the lanes containing proteins of macrophages infected with *Leishmania* are observed to be labeled (Figure 7). This may be due to the failure of [ $^{35}$ S]-methionine to penetrate the lysosomal compartments of the macrophage (observations of Dr. B. Finlay).



**Figure 7.** Autoradiogram of  $^{35}\text{S}$ -methionine-labeled murine BMM infected with *L. donovani*.

Cells were either untreated (lane 1), infected with *Leishmania* and incubated at  $37^{\circ}\text{C}$  for either 6 or 24 hr (lanes 2 and 3, respectively), exposed to a suspension of *S. aureus* for 24 hr (lane 4), treated with IFN- $\gamma$  (1000 U/ml for 36 hr) (lane 5) or incubated with 40  $\mu\text{M}$  of  $\text{NaAsO}_2$  for 3 hr (lane 6). Cells were then pulsed with Translabel [ $^{35}\text{S}$ ]-methionine for 1.5 hr, solubilized in SDS sample buffer and subjected to 10% SDS-PAGE and autoradiography. Molecular weight markers are shown in the margin.



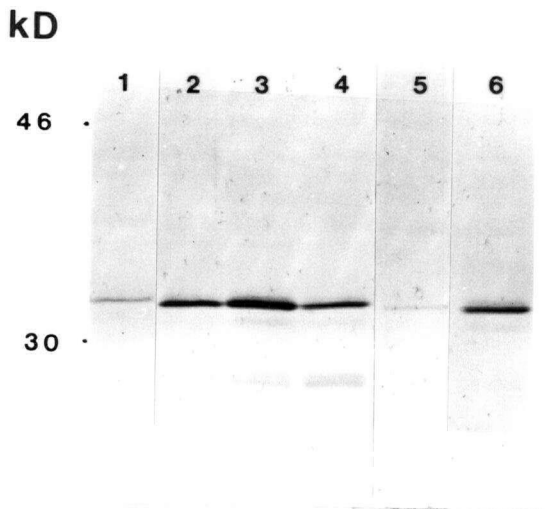
**Figure 8.** Autoradiogram of  $^{35}\text{S}$ -methionine labeled murine BMM infected with *L. donovani*.

Cells were either untreated (lane 1), infected with *Leishmania* and incubated at  $37^{\circ}\text{C}$  for either 24, 12, 6 or 3 hr (Lanes 2, 3, 4 and 5, respectively), exposed to a suspension of *S. aureus* for either 24, 12, 6 or 3 hr (lanes 6, 7, 8, and 9, respectively), treated with  $40\mu\text{M}$  of  $\text{NaAsO}_2$  (lane 10) or heat shock at  $42^{\circ}\text{C}$ , 40 min (lane 11). Cells were then pulsed with Translabel [ $^{35}\text{S}$ ]-methionine for 1.5 hr, solubilized in SDS sample buffer and subjected to SDS-PAGE and autoradiography. Molecular weight markers are shown in the margin.

Additional experiments examined the dynamics of expression of the 32-kDa protein. Metabolic labeling of infected BMM demonstrated that the 32-kDa protein was induced in leishmania-infected BMM as early as 3 hr after infection (Figure 8, lane 5). Thereafter, synthesis of this protein diminished and by 24 hr it was no longer detectable (Figure 8, lanes 2-4). Similarly, synthesis of a 32 kDa protein was also induced between 3 to 12 hr (Figure 8, lanes 7-9), but returned to basal level at 24 hr (Figure 8, lane 6) after phagocytosis of *S.*

*aureus* by BMM. As noted previously, synthesis of 32 kDa protein was also induced by  $\text{NaAsO}_2$  ( $40 \mu\text{M} \times 3 \text{ hr}$ ) (Figure 8, lane 10) and by HS ( $42^\circ \text{C} \times 20 \text{ min}$ ) (Figure 8, lane 11).

Immunoblotting was used to examine whether the  $M_r$  32,000 protein induced in leishmania infected BMM is an inducible form of the 32 kDa HSP heme oxygenase (HO-1). As shown in Figure 9, BMM infected with *L. donovani* and incubated for either 3, 24 or 48 hr (lanes 2-4, respectively) showed enhanced expression of HO-1 with maximum accumulation at 24 hr. This protein was also induced by  $\text{NaAsO}_2$  ( $40 \mu\text{M} \times 3 \text{ hr}$ ) (Figure 9, lane 6), but not by  $\text{IFN-}\gamma$  ( $1000 \text{ U/ml} \times 36 \text{ hr}$ ) (Figure 9, lane 5).



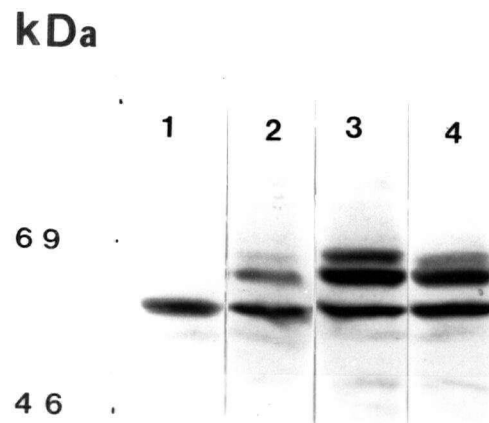
**Figure 9. Anti-heme oxygenase immunoblot of BMM infected with *L. donovani*.**

Cells were either untreated (lane 1) or infected with leishmania amastigotes and incubated at  $37^\circ \text{C}$  for either 3, 24 or 48 hr (lanes 2, 3 and 4, respectively), treated with  $\text{IFN-}\gamma$  ( $1000 \text{ U/ml} \times 36 \text{ hr}$ ) (lane 5), or incubated with  $\text{NaAsO}_2$  ( $40 \mu\text{M} \times 3 \text{ hr}$ ) (lane 6). The remainder of the legend is as for Figure 1. Molecular weight markers are shown in the margin.

#### **b) Regulated expression of novel $M_r$ 65,000 and 67,000 proteins in leishmania-infected BMM**

To examine HSP60 expression in infected cells, BMM were infected with stationary phase promastigotes of *L. donovani*. Promastigotes were used because of the relative ease by which large numbers of these organisms can be obtained in culture medium in a short period of time. Immunoblots performed using rabbit polyclonal antibodies raised against *Heliothis virescens* HSP60 (SPA 805) demonstrated that infection of BMM with *L. donovani* resulted in the expression of 65- and 67-kDa heat-regulated proteins (Figure 10, lanes 2, 3, and 4) that were

not present in control cells. These molecules were coordinately expressed in a time-dependent manner (Figure 10 and Table 1). Both proteins, were detectable at 3 hr of infection (Figure 10, lane 2), reached maximum levels of expression at 24 hr (52% and 100% increases above 3 hr levels respectively for the 65 and 67 kDa proteins) and were coordinately somewhat diminished at 48 hr (Figure 10 lane 4). The anti-HSP60 reagent SPA 805 also recognized an additional protein in both non-infected and infected BMM of  $M_r$  60,000 (Figure 10, lanes 1-4) which presumably represents murine HSP60. In contrast to the  $M_r$  65,000 and 67,000 proteins, expression of murine HSP60 was constitutive and showed no consistent changes with infection.

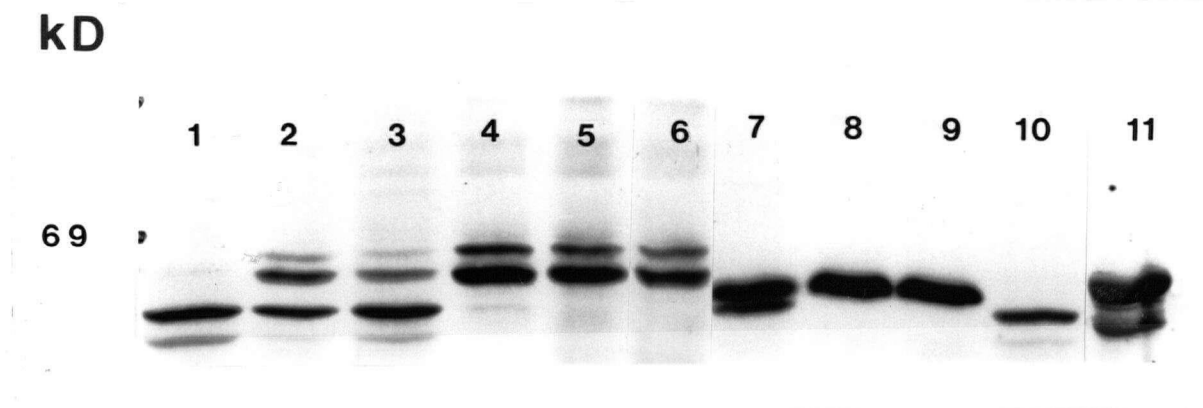


**Figure 10. Anti-HSP60 immunoblot of leishmania-infected BMM.**

Cells were either uninfected (lane 1) or infected with stationary phase promastigotes of *L. donovani* at a ratio of 20 organisms per macrophage and incubated at 37° C for either 3, 24 or 48 hr (lanes 2-4 respectively). After incubation, cells were detached with a cell scraper. Diff-quick stained cell samples indicated an intensity of infection of 94-97 % with  $8.7 \pm 3.6$  (mean  $\pm$  SD) amastigotes per macrophage. Cells were lysed in Laemmli sample buffer followed by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with polyclonal rabbit antibodies to HSP60 (Stressgen) and developed using ECL (Amersham). Molecular weight markers are shown on the left. The data shown are from one of three independent experiments which yielded similar results.

Additional experiments indicated that the  $M_r$  65,000 and 67,000 proteins expressed in infected cells are heat-regulated leishmania proteins. Thus, both of these proteins were

observed to be constitutively expressed in promastigotes cultured *in vitro* at room temperature (Figure 11, lane 4) and were increased in abundance when promastigotes were subjected to heat shock by culture at 37° C for either 3 or 24 hr (Figure 11, lanes 5 and 6, respectively and Table 2). Because promastigotes cultured at 37° C for greater than 24 hr became non-viable, it was not possible to follow the expression of these proteins at 48 hr. Nevertheless, both in infected macrophages (Figure 11, lanes 2 and 3) and in leishmania promastigotes, the expression and modulation of the M<sub>r</sub> 65 and 67 heat-regulated proteins was coordinate both with respect to time and relative abundance (Tables 1 and 2).



**Figure 11. Anti-HSP60 immunoblot of BMM, promastigotes of *L. donovani* and *Y. pseudotuberculosis*.**

Macrophages were uninfected (lane 1), infected with stationary phase promastigotes of *L. donovani* and incubated for either 3 or 24 hr (lanes 2 and 3 respectively), infected for 3 hr with an overnight culture of *Y. pseudotuberculosis* (lane 7) or were exposed to a suspension of fixed *S. aureus* (lane 10). Stationary phase *L. donovani* promastigotes were cultured at either room temperature or 37° C for 24 hr (lanes 4 and 6 respectively) or were incubated at room temperature for 21 hr and at 37° C for a final 3 hr (lane 5). Broth cultures of *Y. pseudotuberculosis* were incubated overnight either at 30° C (lane 8) or incubated for a final 3 hr at 37° C (lane 9). Lane 11 contains recombinant mycobacterial HSP65 as a reference. The remainder of the legend is as for Figure 10. Molecular weight markers are shown on the left. The data shown are from one of two independent experiments that yielded similar results.

To provide further evidence that these two heat-inducible proteins were in fact leishmania in origin and that they were not macrophage-derived in response to the stress of infection, parallel cultures of BMM were infected with viable *Y. pseudotuberculosis* or were allowed to

ingest inactivated *S. aureus*. As shown in Figure 11, neither infection of macrophages with *Y. pseudotuberculosis* (lane 7) nor phagocytosis of *S. aureus* (lane 10) brought about the expression of the  $M_r$  65,000 and 67,000 proteins detected in leishmania-infected BMM (Figure 11, lanes 2 and 3). Of interest, infection of cells with *Y. pseudotuberculosis* resulted in the appearance of a relatively abundant protein (Figure 11, lane 7, upper band) which was not present in either control or leishmania-infected cells. This protein appeared to represent a constitutive form of yersinia HSP60 as it was detected in cultures of *Y. pseudotuberculosis* alone and its expression was not temperature-dependent (Figure 11, lanes 8 and 9).

**Table 1. Expression of HSP60-related proteins in bone marrow-derived macrophages infected with *L. donovani* <sup>1</sup>**

$M_r$	duration of infection (hr)		
	3	24	48
	(integrated optical density units) <sup>2</sup>		
67,000	0.9 $\pm$ 0.3	1.8 $\pm$ 1.3	1.6
65,000	2.1 $\pm$ 0.7	3.2 $\pm$ 1.3	2.4 $\pm$ 1.4
60,000	3.3 $\pm$ 0.7	2.9 $\pm$ 0.7	2.9 $\pm$ 1.0

<sup>1</sup> Monolayers of BMM were infected with stationary phase promastigotes of *L. donovani* at a ratio of 20 parasites per macrophage. Infected cells were incubated at 37° C for either 3, 24 or 48 hr, and then detached mechanically and assessed for intensity of infection as indicated in the legend to Figure 10. Infected cells were washed with warm HBSS, solubilized in Laemmli sample buffer and proteins (60 ug per lane) were separated by 10% SDS-PAGE. Separated proteins were then transferred to nitrocellulose membranes and immunoblotted with polyclonal rabbit antibodies to HSP60. The resulting bands were scanned for determination of optical density units (Video Densitometer 620, BioRad). The level of expression of the  $M_r$  60,000 protein (HSP60) in control non-infected cells was 3.1  $\pm$  0.7.

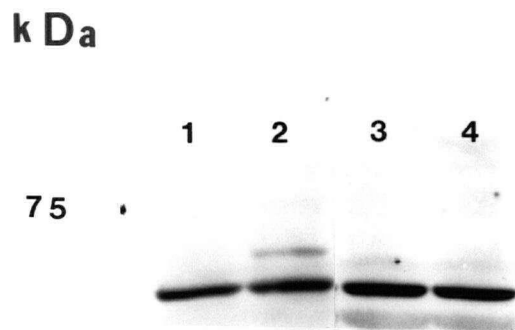
<sup>2</sup> The data shown are the mean  $\pm$  SD of values obtained in three independent experiments which used different batches of BMM.

**Table 2. Expression of HSP60-related proteins in promastigotes of *L. donovani* <sup>1</sup>**

$M_r$	duration of culture at 37° C (hr)		
	0	3	24
	(Integral optical density units) <sup>2</sup>		
67,000	1.8	3.0	2.7
65,000	3.3	4.3	4.0

<sup>1</sup>Stationary phase promastigotes of *L. donovani* growing in Senekjie medium were harvested, washed three times in HBSS and resuspended in RPMI containing 10% fetal bovine serum (FBS) penicillin (100 U/ml) and streptomycin (100 ug/ml), and then incubated either for 3 or 24 hr at 37° C. After incubation, motile promastigotes were adjusted to a concentration of  $4 \times 10^8$ /ml and solubilized in Laemmli Sample buffer. SDS-PAGE, Western blotting and densitometry were carried out as indicated in the legend to Table 1.

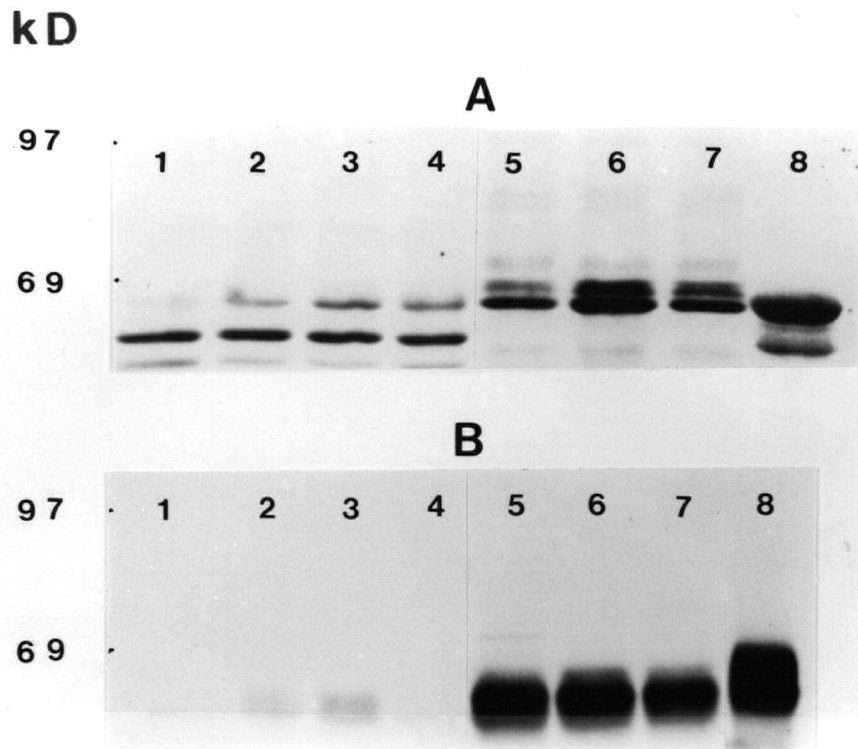
<sup>2</sup> The results shown are the average of values obtained in two separate experiments.

**Figure 12. Anti-HSP60 immunoblot of BMM.**

Cells were either untreated (lane 1), infected with leishmania promastigotes for 24 hr (lane 2), exposed to NaAsO<sub>2</sub> (40 uM for 3 hr) (lane 3) or heat shock (42° for 20 min) (lane 4). After treatment cells were allowed to recover at 37° C for 1 hr and were then processed for Western blotting as indicated in legend to Figure 10. Molecular weight markers are shown on the left. The data shown are from one of two independent experiments that yielded similar results.



Additional evidence shown in Figure 12 indicated that the  $M_r$  65,000 and 67,000 heat-regulated proteins that appeared *de novo* in infected macrophages were not the result of a nonspecific stress response. Thus, neither of these proteins were detected when non-infected macrophages were exposed to either heat shock or  $\text{NaAsO}_2$ . Western blot analysis using the rabbit polyclonal anti-moth HSP60 antiserum detected murine HSP60 in all treatment groups. A protein of  $M_r$  65,000 was also detected in leishmania infected macrophages (Figure 12, lane 2) which corresponded to the smaller and more abundant of the two heat-regulated proteins expressed during leishmania infection (Figures 10 and 11). However, the  $M_r$  65,000 protein was not detected in cells exposed to either  $\text{NaAsO}_2$  or heat shock (Figure 12, lanes 3 and 4 respectively) even though both treatments brought about modest increases in the expression of murine macrophage HSP60 (Figure 12, lanes 3 and 4).



**Figure 13. Anti-HSP60 (panel A) and anti-gp63 (panel B) immunoblots of leishmania infected BMM and promastigotes of *L. donovani*.**

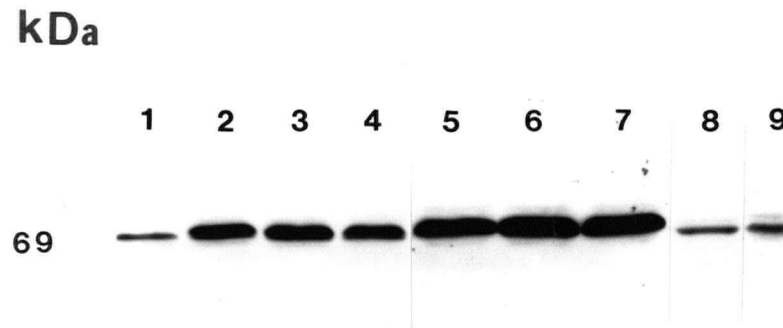
Macrophages were either uninfected (lanes 1, both panels) or infected with stationary phase promastigotes and then incubated for either 3, 24 or 48 hr (lanes 2-4, respectively, both panels). Stationary phase promastigotes were incubated for 24 hr at either room temperature (lanes 5, both panels) or 37 ° C (lanes 6, both panels) or were incubated at room temperature for 21 hr and for a final 3 hr at 37 ° C (lane 7, both panels). Lanes 8 contained either recombinant mycobacterial HSP65 (panel A) or recombinant leishmania gp63 (panel B). The remainder of the legend is as for Figure 10. Molecular weight markers are shown on the left. The data shown are from a single experiment.

Experiments were also carried out to examine whether the abundant, regulated expression of the two leishmania heat-regulated proteins was a specific event or whether this might be a general phenomenon applicable to other leishmania (non-stress) proteins. As shown in Figure 13, comparison of the  $M_r$  65,000 and 67,000 leishmania heat-regulated proteins with that of the abundant leishmania surface protease gp63 in BMM demonstrated that expression of the leishmania heat-inducible proteins was selectively upregulated during infection. In the experiment shown, only the smaller, more abundant  $M_r$  65,000 leishmania protein was observed to be expressed in a time-dependent manner in infected BMM (Figure 13A, lanes 2-

4). When extracts from the same cells were subjected to immunoblotting with a monoclonal antibody specific for leishmania gp63, initial low level expression of the leishmania surface protease was apparent, (Figure 13B, lanes 2 and 3). In contrast to the heat-regulated  $M_r$  65,000 protein of *L. donovani*, however, expression of gp63 decreased markedly and was no longer detectable at 48 hr, at a time when expression of the  $M_r$  65,000 leishmania protein was still high. Time-dependent down-regulation of gp63 expression within infected cells correlated with a progressive, albeit modest, decrease in the expression of gp63 by promastigotes subjected to heat shock during culture at 37° C for either 3 hr or 24 hr (Figure 13B, respectively lanes 7 and 6).

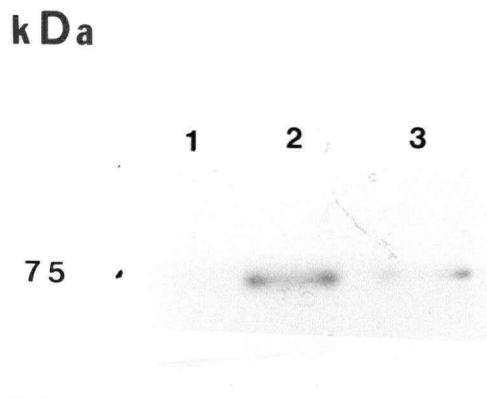
#### **c) Expression of HSP70 in leishmania-infected BMM**

To examine whether other leishmania HSP are also expressed during infection, lysates of infected BMM were analyzed by immunoblotting with murine monoclonal antibody D4F18, raised against a recombinant 72 kDa protein from *P. falciparum* (266). This antibody which is known to recognize both mammalian HSP70 and HSP70 from *Plasmodia* (P. Dubois, O. Bensaude and L. Pereira Da Silva, unpublished data), detected a protein with an apparent subunit  $M_r$  of 70,000 in control cells (Figure 14, lane 1). This presumably represents a constitutive form of mammalian HSP70. Of interest, in comparison to control cells antibody D4F18 showed markedly increased reactivity also with a protein of approximate subunit size of  $M_r$  70,000 in BMM infected with *L. donovani* for either 3, 24 or 48 hr (Figure 14, lanes 2-4). The expression of this protein appeared to be maximal at 3 hr, was stable through 24 hr and appeared to decrease slightly by 48 hr post-infection.



**Figure 14. Anti-HSP70 immunoblot of leishmania infected BMM and promastigotes of *L. donovani* performed using monoclonal antibody D4F18 specific for a constitutive form of HSP70.**

Macrophages were either untreated and cultured for 48 hr (lane 1) or were incubated with stationary phase promastigotes and then cultured for either 3, 24 or 48 hr (lanes 2-4, respectively). Stationary phase promastigotes were incubated for 24 hr at either room temperature (lane 5) or 37° C (lane 7) or were incubated for 21 hr at room temperature and for a final 3 hr at 37° C (lane 6). Lane 8 shows BMM exposed to fixed *S. aureus* and macrophages exposed to NaAsO<sub>2</sub> (40 uM for 3 hr) are shown in lane 9. Integrated optical density units for lanes 1-9 respectively were: 1.8, 4.0, 4.0, 3.5, 5.2, 6.0, 6.2, 1.9, and 1.8. The remainder of the legend is as for Figure 10. Molecular weight markers are shown on the left. The data shown are from a single experiment.



**Figure 15. Anti-HSP70 immunoblot of stressed macrophages.**

Cells were untreated (lane 1) or exposed to either NaAsO<sub>2</sub> (40 uM for 3 hr, lane 2) or heat shock (42° C for 20 min, lane 3). The remainder of the legend is as for Figure 10 except that the anti-HSP70 monoclonal antibody (SPA 810, Stressgen) specific for the inducible form of the HSP70 was used in lieu of D4F18. Molecular weight markers are shown on the left. The data shown are from one of three independent experiments that yielded similar results.

Three findings indicate that this protein likely represents leishmania HSP70 and not an inducible form of mammalian HSP70. First, antibody D4F18 also detected a protein with an identical subunit size which was expressed constitutively in promastigotes cultured at 25° C (Figure 14, lane 5) and which appeared to be modestly upregulated during promastigote culture at 37° C for either 3 hr or 24 hr (Figure 14, lanes 6 and 7). Second, when non-infected BMM were stressed either by phagocytosis of inactivated *S. aureus* or by treatment with 40 uM NaAsO<sub>2</sub> for 3 hr (Figure 14, lanes 8 and 9 respectively), in comparison to control cells there was no change in signal intensity generated using antibody D4F18. Third, when BMM were stressed by either heat shock or NaAsO<sub>2</sub> and lysates were examined using an anti-HSP70 monoclonal antibody (SPA 810, Stressgen) that specifically recognizes an inducible form of the HSP70, a protein with a higher apparent subunit  $M_r$  of 73,000 was detected (Figure 15, lanes 2 and 3 respectively). This 73 kDa protein was not detected either in control cells in the basal state (Figure 15, lane 1) or in leishmania infected macrophages (data not shown) and presumably represents the inducible form of mammalian HSP70, which is known to have a size in the range of  $M_r$  72,000.

#### 4. Discussion

Infection of cells by intracellular pathogens represents a form of stress that affects both the invading organism and the host cell. These interactions may bring about a stress response and induction of HSP expression (2,44,106). There is only limited direct information available about the expression of either host- or pathogen-derived HSPs within infected cells. For example, it has been observed that both the GroEL and DnaK proteins of *Salmonella spp* are expressed within infected macrophages. It has also been shown that normal murine BMM contain a 68 to 70 kDa protein that is recognized in Western blots by monoclonal antibody IA10, specific for mycobacterial HSP65. Furthermore, when BMM are infected with *M. bovis*, expression of mycobacterial HSP65 is also detectable in infected cells and this protein can be distinguished from the cross-reactive murine macrophage 68 to 70-kDa homolog (142). In addition, monoclonal antibody, IA10, also recognizes a 65-kDa protein induced in

peritoneal macrophages from *T. gondii*-infected mice which is not expressed in cells from corresponding control animals (168). More recently, monoclonal antibody 7.10 raised against *Drosophila* HSP70 was found to recognize leishmania HSP70, but not host HSP70 in human alveolar macrophages infected with *L. major* (291).

In the present series of experiments the expression of HSPs was examined in BMM infected with leishmania. BMM were infected with amastigotes of *L. donovani* and then subjected to metabolic [ $^{35}\text{S}$ ]-labeling. Cell lysates were analyzed by both SDS-PAGE and autoradiography Western blotting using anti-HSP specific antibodies. Analysis of autoradiographs indicated that synthesis of a 32 kDa protein is prominently induced in leishmania infected macrophages. Increased synthesis was detectable as early as 3 hr after infection. Thereafter, synthesis of the 32-kDa protein diminished such that by 24 hr it was no longer detectable. Similarly, increased production of a 32 kDa protein was also observed when cells were treated with either NaAsO<sub>2</sub> or heat shock or during phagocytosis of *S. aureus* by BMM. Immunoblotting using a mAb antibody raised against rat liver heme oxygenase-1 (HO-1) indicated that this enzyme, which is also 32 kDa and commonly induced during oxidative stress (91), is also induced in leishmania infected BMM. Additional experiments indicated that levels of HO-1 are increased in leishmania infected BMM at 3, 24 and 48 hr of incubation while the constitutive level of this enzyme remained unchanged in noninfected cells. Furthermore, treatment of BMM with NaAsO<sub>2</sub>, a known inducer of HO-1 (85) also brought about increased HO-1 expression in BMM as determined by immunoblotting. These findings indicate that HO-1 is induced in leishmania infected BMM and suggest that the 32 kDa protein detected by metabolic labeling is HO-1.

The question of the origin of HO-1 expression in infected macrophages was not addressed in these experiments. Two observations, however, strongly suggest that HO-1 expression in leishmania infected BMM is of host origin rather than being pathogen-derived. First, NaAsO<sub>2</sub> treatment of BMM induces HO-1 and second, phagocytosis of fixed *S. aureus* induces a similar 32 kDa protein in BMM as determined by [ $^{35}\text{S}$ ]-methionine labeling. In this regard, it has also been shown that phagocytosis of either inactivated *S. aureus* or sheep red blood cells

by human macrophages induces HO-1 (80) indicating that HO-1 induction in macrophages during phagocytosis may be common. In this context, HO-1 is likely involved in protection of cells against the oxidative stress brought about by infection. The protective properties of HO-1 are likely related to its ability to cleave the heme molecule to produce bilirubin and biliverdin, both of which are effective scavengers of ROI (91).

Analysis of autoradiographs of SDS-PAGE gels prepared from cells labeled with [ $^{35}\text{S}$ ]-methionine indicated that proteins with mobilities consistent with HSP90, HSP70 or HSP60 were not induced in leishmania infected cells (Figs. 7 and 8). The findings that expression of these host HSPs was not induced in *L. donovani* infected cells is consistent with recent data indicating that infection of human alveolar macrophages with *L. major* also did not result in the induction of host HSPs (291).

In contrast to the absence of induction of these host HSPs, when lysates of leishmania-infected BMM were analyzed by immunoblotting using antibodies to HSPs, it was found that leishmania HSP60 and HSP70 are expressed and induced in infected cells. Of particular interest were the findings related to leishmania HSP60. Two proteins recognized by anti-HSP60 antibodies appeared de novo in infected BMM and these proteins were found to be leishmanial in origin. These proteins with subunit molecular sizes of 65- and 67-kDa, appeared rapidly (within 3 hr of infection) and were inducible with maximal expression observed at 24 hr.

That these two proteins are indeed leishmania HSPs and not novel forms of murine HSP60 is supported by two observations. First, two proteins with identical electrophoretic mobilities (65- and 67-kDa) and in the same relative abundance were demonstrated in stationary-phase leishmania promastigotes. Moreover, these two proteins which were observed to be expressed constitutively in promastigotes cultured at room temperature, demonstrated the same kinetics of inducibility in response to heat shock as did the 65- and 67-kDa proteins detected in infected BMM. Second, these proteins were not detected in noninfected cells which had been exposed to several conditions known to induce stress protein expression, including (i) infection with *Y. pseudotuberculosis*, (ii) phagocytosis with *S. aureus*, (iii) NaAsO<sub>2</sub> treatment, and (iv) heat

shock. It is also of note that the abundant expression of the 65- and 67-kDa heat-regulated leishmania proteins was selective, in that expression of the leishmania surface protease gp63 within infected cells was observed to decrease at a time when expression of these heat-regulated leishmania proteins was increasing.

Infection of BMM with *L. donovani* was also accompanied by the expression of what appeared to be a minimally inducible leishmania protein of the HSP70 family. This protein has an apparent subunit size of 70-kDa and was thus identical in size to murine HSP70. The latter was also recognized by the anti HSP-70 monoclonal antibody D4F18, raised against HSP70 from *P. falciparum* and was expressed constitutively in noninfected cells. The expression of this murine HSP70 did not change significantly in response to either treatment with NaAsO<sub>2</sub>, phagocytosis of *S. aureus*, or infection with *L. donovani*. Thus, it appears to correspond to the 70-kDa mammalian heat shock cognate protein which is known to be noninducible (1,44,106). Moreover, an approximately 73-kDa protein not detectable in the basal state and which was strongly induced in response to both NaAsO<sub>2</sub> treatment and heat shock, was observed in immunoblots of normal BMM performed with a monoclonal antibody specific for the inducible form of mammalian HSP70 (known to be ~72 kDa in size). As was the case for the 65- and 67-kDa *Leishmania* proteins, leishmania HSP70 was also expressed constitutively in stationary-phase promastigotes and was observed to be modestly inducible. On the basis of these findings, it is concluded that leishmania HSP70 is induced to a modest extent in infected cells, whereas the expression of mammalian HSP70 is unchanged.

The data presented above indicate that in contrast to host HO-1 and the leishmania 65- and 67-kDa heat-regulated proteins, host proteins belonging to the 60-kDa, 70-kDa and 90-kDa HSP families do not appear to alter their expression in response to infection. This finding is of significant interest, as it is well known, for example, that viral infections frequently bring about increased expression of HSPs and it has been postulated that other intracellular infections with bacteria as well as with more complex organisms may do so as well (44). At least with respect to *L. donovani* and members of the murine HSP60, HSP70 and HSP90 families, this appears not to be the case.



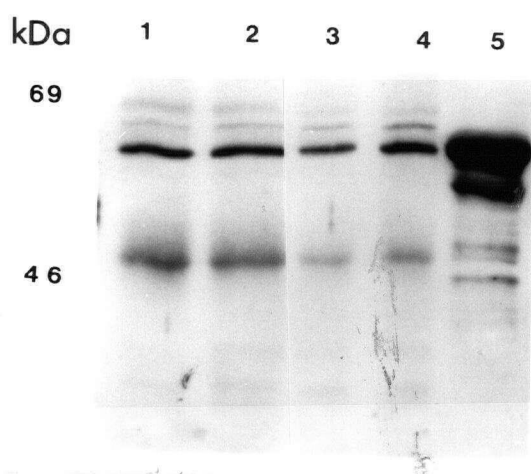
The HSP70 gene from *L. donovani* has been cloned and found to be present in the genome on a single chromosome in multiple tandem repeats (131). The corresponding mRNA and protein for this gene are both constitutively expressed and only modestly induced by heat in either promastigotes or amastigotes. The expression of this protein also does not change significantly during conversion from amastigotes to promastigotes in culture. The 70 kDa HSP observed to be expressed in infected BMM in this study also had limited inducibility and likely corresponds to *L. donovani* HSP70 (131). Moreover, in respect to its modest inducibility, this HSP likely represents the leishmania homolog of the 70-kDa heat shock cognate protein described for other organisms. While little is known about the immune response to this protein, its abundant expression in infected cells suggests that it may be of significance and this is consistent with the finding that serum samples from 50% of patients with visceral leishmaniasis recognize a fusion protein containing HSP70 from *L. donovani* (131). Furthermore, B cell epitope mapping using sera from infected patients demonstrated that the immunodominant site of leishmania HSP70 is located in the carboxy-terminal region, the most evolutionarily divergent part of the molecule (257). This is consistent with the findings indicating that sera from patients with either cutaneous leishmaniasis, Chagas' disease, leprosy, malaria or schistosomiasis do not recognize recombinant HSP70 from *L. donovani* (257). Thus, the immune response to this protein may be specific.

In contrast to HSP70, relatively little information is available about the HSP60 family in *Leishmania spp.* It is known from metabolic labeling studies of leishmania promastigotes that during exposure to either NaAsO<sub>2</sub> or heat shock, increased expression of several proteins, including proteins in the 60- to 70-kDa range is observed (242). Prior to the work described in this thesis, cloning and sequencing of the leishmania HSP60 gene had not been reported. From the findings of the present study, it is clear that at least two HSPs of 65 and 67 kDa are present in *L. donovani* and that both of these are relatively abundantly expressed in infected macrophages. In the context of what is known from other systems of intracellular infection, it seems likely that this may have important implications for the immune response to leishmania infection. Results obtained with *M. tuberculosis*, *M. bovis*, and *M. leprae* indicate that the

mycobacterial HSP65 is immunogenic in vivo and that it elicits both T- and B-cell responses (106). Furthermore, recent findings indicate that immunization with HSP60 from either *L. pneumophila* (167), *Y. enterocolitica* (167), *M. leprae* (170), *H. pylori* (171) confers high levels of protection against infection by these organisms in animal models. This raises the possibility that the 65- and 67-kDa leishmania HSPs may be involved in protection against leishmania infection.

## B. CLONING, SEQUENCING, EXPRESSION AND PURIFICATION OF rLHSP60

The findings that infection of macrophages with *L. donovani* results in the expression of two heat regulated leishmania proteins belonging to the HSP60 family, raised the possibility that these proteins may be involved in the immune response to leishmania. In order to facilitate further studies of these proteins the leishmania HSP60 gene was cloned from *L. major*, sequenced and expressed in *E. coli*. *L. major* was chosen because of the availability of a  $\lambda$  EMBL-3 *L. major* genomic library for screening and because the 65- and 67-kDa proteins observed to be expressed in *L. donovani* infected BMM were also expressed in *L. major* promastigotes cultured at either 27<sup>o</sup> C or 37<sup>o</sup> C (Figure 16).

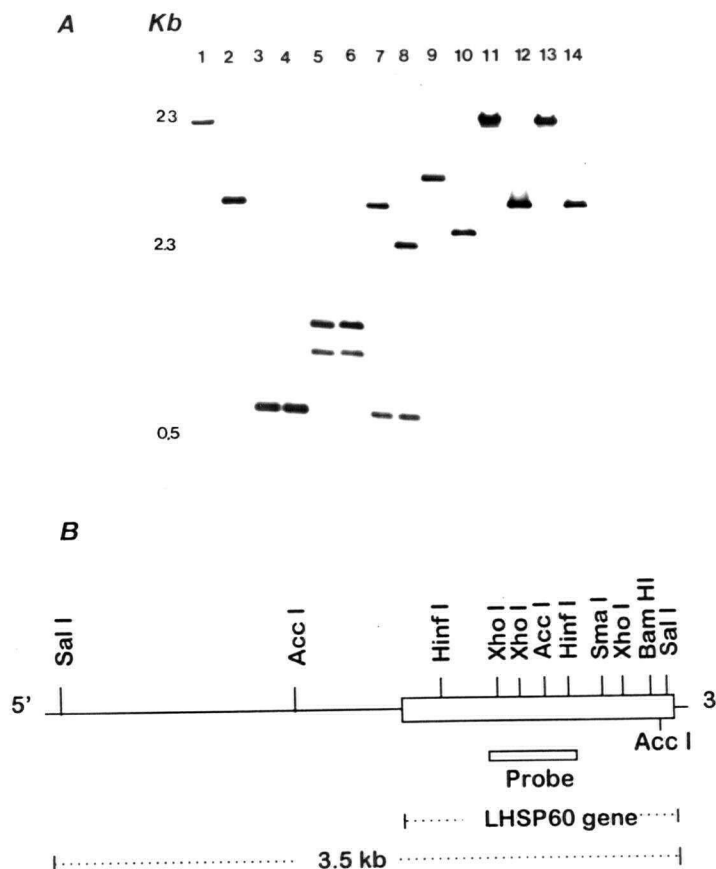


**Figure 16. Anti-HSP60 immunoblot of *L. donovani* (lanes 1 and 2) and *L. major* (lanes 3 and 4).**

Stationary phase promastigotes were untreated (lanes 1 and 3) or exposed to heat shock (37<sup>o</sup> C for 3 hr, lanes 2 and 4). After treatment, cells were washed in HBSS and processed for immunoblotting (as in Figure 10) using the anti-HSP60 polyclonal antibody raised against *Cyanobacteria* (SPA 806, Stressgen). Recombinant mycobacterial HSP65 is shown in lane 5. Molecular weight markers are shown on the left.

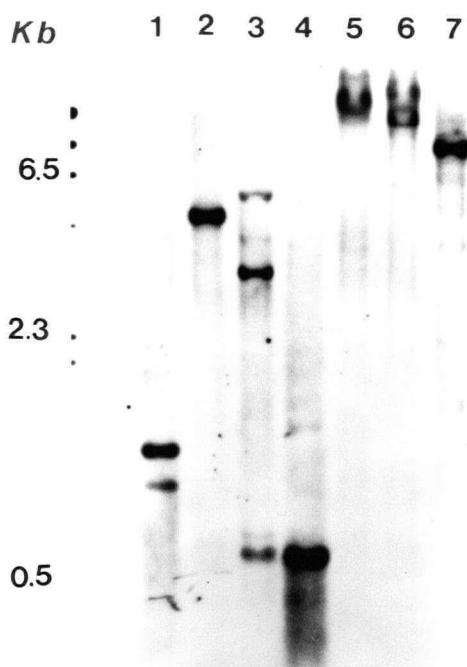
### 1. Cloning and sequencing the *L. major* HSP60 gene.

A DNA probe prepared by PCR using two highly conserved regions in the HSP60 family of proteins (see Materials and Methods) was used to screen a  $\lambda$ EMBL-3 *L. major* genomic DNA library. A single clone, designated 232, that hybridized strongly with the HSP60 DNA probe was identified, plaque purified twice and found to contain a 17 Kb insert. Analysis of this insert by restriction enzyme (RE) digestion and Southern blotting identified a unique *Sal* I fragment of 3.5 Kb (Figure 17A, lane 2) that hybridized with the HSP60 DNA probe. Further RE analysis of the *Sal* I fragment established that it contained a 1.7 Kb fragment that appeared to contain the *L. major* HSP60 gene (Figure 17B). Analysis of restriction endonuclease digests of (i) the 17 Kb insert from  $\lambda$ EMBL-3 clone 232 (Figure 17), and (ii) *L. major* genomic DNA (Figure 18) revealed that these produced identical patterns in Southern blots with the HSP60 DNA probe. Thus, in both genomic and clone 232 DNA, digestion with *Acc* I generated fragments of 1.0 and 0.7 Kb, *Xho* I generated fragments of 0.6 and 3.7 Kb, *Sma* I produced a single fragment of ~5 Kb, and *Bam* HI, *Hind* III and *Eco* RI each generated single fragments of 9, 23, and 23 Kb, respectively (Figure 17 and 18). This analysis indicated that the HSP60 gene appears to be present as a single copy gene.



**Figure 17. Southern blot (A) and restriction endonuclease map (B) of *L. major* Sal I fragment containing the *L. major* HSP60 gene.**

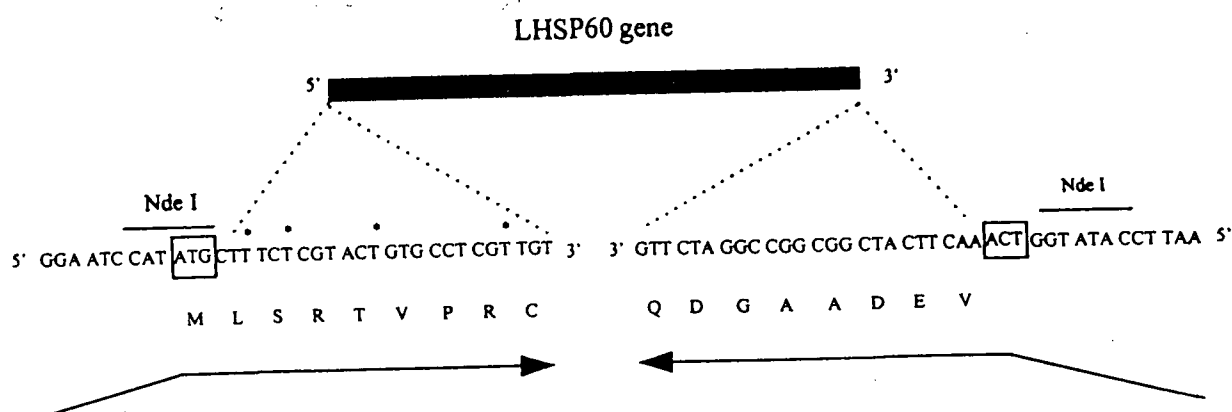
(A) DNA (1 ug) from clone 232 was subjected to single digestion with either *Bam* HI (lane 1), *Sal* I (lane 2), *Hinf* I (lane 3), *Acc* I (lane 5), *Xho* I (lane 7), *Sma* I (lane 9), *Hind* III (lane 11), *Eco* RI (lane 13) or to double digestion with combinations of *Sal* I + *Hinf* I (lane 4), *Sal* I + *Acc* I (lane 6), *Sal* I + *Xho* I (lane 8), *Sal* I + *Sma* I (lane 10), *Sal* I + *Hind* III (lane 12) or *Sal* I + *Eco* RI (lane 14). Digested DNA was fractionated in 1% agarose gels, transferred to positively charged nylon membranes and hybridized with the HSP60 probe that was used for screening the  $\lambda$ EMBL-3 library. Size standards are  $\lambda$  DNA fragments cleaved with *Hind* III and labeled with DIG. The Southern blot was developed by chemiluminescence. (B) Restriction endonuclease map of clone 232. The *Sal* I fragment, the location of the leishmania HSP60 gene on the 3' end of the fragment and the location of the PCR probe within the gene are shown.



**Figure 18. Southern blotting of *L. major* genomic DNA.**

Genomic DNA (5 ug) from *L. major* was digested with either *Acc* I (lane 1), *Sma* I (lane 2), *Xho* I (lane 3), *Hinf* I (lane 4), *Hind* III (lane 5), *Eco* RI (lane 6) or *Bam* HI (lane 7), fractionated on a 1% agarose gel, transferred to a positively charged nylon membrane, and hybridized with the HSP60 probe. The Southern blot was developed by chemiluminescence. Size standards are  $\lambda$  DNA fragments cleaved with *Hind* III and labeled with DIG.

Given the potential for gene rearrangement during construction of the  $\lambda$ EMBL-3 genomic library, it was necessary to confirm that the *Sal* I fragment contained the authentic HSP60 gene. To accomplish this PCR was carried out using primers that included start and termination codons (Figure 19) and either *L. major* genomic DNA or clone 232 DNA as templates. The PCR products from leishmania genomic DNA and from clone 232 were identical in size at  $\sim 1.7$  Kb (data not shown), which was also the expected size of the leishmania HSP60 gene as judged by the DNA sequence. Restriction enzyme analysis using DNA from clone 232 indicated the presence of a *Bam* HI site at the 3' end of the LHSP60 gene (Figure 17). Based upon the nucleotide sequence, *Bam* HI is expected to digest the gene into a 5' fragment of 1473 bp and a 3' fragment of 297 bp. Indeed, when the PCR products from either leishmania genomic DNA or from clone 232 were digested with *Bam* HI, fragments of  $\sim 1.4$  kb and  $\sim 0.3$  kb were obtained (not shown). These findings provide strong evidence to indicate that the PCR products obtained from leishmania genomic DNA and from clone 232 DNA were identical.



**Figure 19.** Strategy used to amplify the *LHSP60* gene from  $\lambda$ EMBL-3 clone 232 by PCR for cloning into the pET-3a vector and expression in *E. coli*.

Two oligonucleotide primers were designed from the initiation and termination sequences of the gene (designated by the arrows below the respective sequences). Nucleotide sequences and the translated protein sequences for each primer are shown. Modified nucleotides on the 5' end of the gene (asterisks) are also shown. *Nde* I sites were included in both primers, at the ATG-start and at the TGA termination codons. The 5' ends of each primer (sloped segments on the tails of each arrow) are not HSP60 sequences, but contain the *Nde* I restriction site. Translation start and stop sites are boxed.

Both strands of the *Sal* I fragment from clone 232 subcloned into the pGem-3Zf (+) sequencing vector were sequenced. Sequence analysis revealed the presence of an open reading frame of 1770 bp encoding a putative polypeptide of 589 amino acids with a predicted size of  $M_r$  64,790 (Figure 20) possibly corresponding to the leishmania *HSP60* gene.

An alignment of the *L. major* HSP60 protein sequence with other eukaryotic and prokaryotic HSP60 sequences is shown in Figure 21. Positions of identical and conserved residues are indicated in the alignment. Similar to *T. cruzi* (292), *L. major* HSP60 also contains a short mitochondrial targeting sequence (MLSRTVPRCVKYGST) located at the N-terminus. This analysis also demonstrates that in contrast to most other HSP60 homologs, the *L. major* sequence does not contain the repeat GGMGGM motif located between residues 546-573 at the C-terminus (Figure 21).

ATGCTCTCCGTCAG GTGCTCGCTGTGTC AAGTACGGCTCAACG CCGAAGGACATCCGC TATGGCATGGAGGCC CGCAACGCCCTGCTT GCCGGCGTCGAGAAC CTTGTCAAGGCCGTC 120  
 M L S R T V P R C V K Y G S T P K D I R Y G M E A R N A L L A G V E N L V K A V  
 GCGTCACGCTGGGC CCCAAGGGCCGCAAC GTGATTCTGGAGTG CCATACGCATGCCG AAGATCAGCAAGGAT GGAGTCACGGTGGCC AAGAGCATCGAGTTC GAGGACAGCTTCGAG 240  
 G V T L G P K G R N V I L E M P Y A C P K I T K D G V T V A K S I E F E D S F E  
 AACCTTGGGGCAAC TTGGTCCGTCAGGTG GCAGGGCTGACCAAC GACAACGCCGGTGAC GGCACCACCACCGCT ACTGTCTCTCCGGG GCGATCTTCAAGGAG GGCTTCCGACGGTT 360  
 N L G A N L V R Q V A C L T N D N A G D G T T T A T V L S G A I F K E G F R S V  
 GCAAGTGGCAGAAC CCGATGGACCTGAAG CGGGGCATCGACCTC GCATGCCGGGAGGTG CTCATATCGCTGGCC GAGCAGTCTCGCCA GTCACCTCCAAATCT GAGATCACCAGGTA 480  
 A S G T N P M D L K R G I D L A C R E V L I S L A E Q S R P V T S K S E I T Q V  
 GCGATGATTTGGCG AACATGGACCAAGAG ATCGGCAGCTGATC GGTGACCGGATGAG CAGGTGCGCAAGGAT GCGTAATCACAACG CAGGAGGGTGGCTCG CTTAATACGGAGTC 600  
 A M I S A N M D Q E I G S L I G D A M Q Q V G K D G V I T T Q E G R S L N T E L  
 GAGCTGGTCAGGGG ATGCTCTTTGAGCGT GGGTACACGTGCGCG TACTTTGTGACGAAC ACGAAGGCGCAAGG TCGAGCTCGAGAAC GCCCTCGTCTACGTG GCGAACCGCAAATC 720  
 E L V E G M S F E R G Y T S P Y F V T N T K A O R C E L E N A L V Y V A N R K L  
 ACGAGCGTGGCGAC ATCTTCCCGCGCTG AACTACGCCATTGAG CAGAAGCGTCCGTT CTGGTATTGCCGAG GACGTGGAGGGGAG GCCATGCACAGTTC CTGTACAACAAGTT 840  
 T S V A H I L P A L N Y A I Q D K R V T V S R D E C I L M E G G G S A I A V E E R  
 CAGGGCCGATCAGT CGCTGCGCGCTCAAG GCCCGGGCTTGGC GACATGCGCATCAAC CAGTGCAGGATATC GCCGTCTTACGGGG TCGCAGATGATCTCA GAAGACCTAGGCCIT 960  
 Q G R I S G C A V K A P G F G D M R I N Q L D D I A V F T G S Q H I S E D L G L  
 TCATGGACCAAGAC GATTTCCTCGAGCGG TTCTCGGCACTTGT CGAAGGTGACCGTC TCCCGTGACGAGTGC ATCTTGATGGAGGGC GCGGCGAGCGGCATC GCGGTGGAGGAGCA 1080  
 S L D Q N D F S E R F L G T C R K V T V S R D E C I L M E G G G S A I A V E E R  
 GTGCAGATGATCAAA GACATGATCTCCGC GAGGACCACGAATAC AATCGCGAGCGTGT GTGAGCGTCTCGCG AAGCTCTCGGGTGGG GTTGGCGTATCAAG GTCGGTGGCGCTCG 1200  
 V D M I K D M I S A E D H E Y N R E R L V E R L A K L S G G V A V I K V G G A S  
 GAGGTGGAGATCAAT GAGAAGAAGGACCG ATCATCGACGCGCTC AACGCCACGCGCGC GCTGTATCAGAGGGC ATCCTCGCAGGTGGT GGCACCGGGTTGCTT ATGGCTCTCTGCGT 1320  
 E V E I N E K K D R I I D A L N A T R A A V S E G I L A G G G T G L L M A S L R  
 CTCGAGAGCATCTCC AAGGACCGACGACTT CCACCTGACATCCG ACTGGCGTGAACATT GTGAAGAAGGCAATC GGCCTGCCCGCGGC TACATCGCGAACAAC GCAGGCGTCGAGGGC 1440  
 L E S I S K D R R L P P O I R T G V N I V K K A I G L P A R Y I A N N A G V E G  
 AGCGTGGTGGCGGG AAGTACTCGCGCGC AAGGATCCGAGCTTC GGCTACAACGCGCAG ACCGGCGAGTATGT AATATGTTGAGGCT GGCATTATCGATCCC ATGAAAGTAGTGAAG 1560  
 S V V A G K V L A R K D P S F G Y N A Q T G E Y V N M F E A G I I D P M K V V K  
 TCCGCCGTCGTAAC GCATGCTCCGTGGCG GGCATGATGATTACG ACGGAGCGGCGAGTG GTGGAGAAGGACCTG CTAGGGGAGAGAAA CGCATCGAGGATGAG GGGATGGAGGACAAG 1680  
 S A V V N A C S V A G M M I T T E A A V V E K D L L G R E K R I E D E G M E D K  
 GAGAAGAAGCGCAGC GTCGACAACTGCGC AAGCAGGTAATGAG GCGGGACGACCGAT GCCAAGATGGCGCC GCCGATGAAGTTGA. 1770  
 E K K R S V D K L R K Q V N E A G R T D A Q D G A A D E V

**Figure 20. Complete coding and deduced amino acid sequence for the *L. major* HSP60 gene.**

The putative mitochondrial targeting sequence at the N-terminal end (amino acids 1-15) is underlined. The unique *Bam* HI site at the 3' end of the gene is also shown. Nucleotides are numbered beginning with the translation initiation codon ATG. Numbers on the right correspond to positions of nucleotides within the gene.

PILMAJOR	MLS-RTVPRCVKYGST-----	PKDIRYGMERNALLAGVENLVKAVGVTLGP	46
PITCRUZ	MFR-SA-----ARFA-----	GKEIRFGTEAROSMQKGVORAVSAVATTLGP	40
P1PROHU	MLRLPTVFRQMRPVSRVLAPHL-----TRAY-----	AKDVKFADARALMLQGVDLADAVAVTMGP	57
P1MOUSE	MLRLPTVLRQMRPVSRALAPHL-----TRAY-----	AKDVKFADARALMLQGVDLADAVAVTMGP	57
P1RAT	MLRLPTVLRQMRPVSRALAPHL-----TRAY-----	AKDVKFADARALMLQGVDLADAVAVTMGP	57
P1CHO	MLRLPTVLRQMRPVSRALAPHL-----TRAY-----	AKDVKFADARALMLQGVDLADAVAVTMGP	57
P1MOTH	MFKMYRSPHITRNSFKYLKATNINS-----CRFY-----	AKEVRFPGDVRSLMLEGVDILADADDVTMGP	60
P1CUCURB	MHRFATGLASKARLARNGANQIASRSN---WRRNYA-----	AKDVKFADARALMLQGVDLADAVAVTMGP	64
ARATHMIT	MYRFASNLASKARIAQN-ARQVSSRMS--WSRNYA-----	AKDKFGEARALMLKGVDELADAVKVTMGP	63
MAIZEMIT	MYRAAASLASKARQAGNSLATROVGSRLAWSRNYA-----	AKDKFGEARALMLRGVEELADAVKVTMGP	66
BNAPUSMT	MYRLISSIASKARVARNCTSQIGSRLS--STRNYA-----	AKDIRFGVEGRALMLRGVEELADAVKVTIPP	64
P1ECOLI	MA-----	AKDVKFQNDARVKMLRGVNVVLADAVKVTLGP	33
P1HLEP	M-----	AKTIAYDEEARRGLERGLNSLADAVKVTLGP	32
P1PLAFA	M-RMKRIHILFVVI FLLCLRYGYSIKKRSPPNNKRLFINKRLKYINSKISRRKENYVKMKMTENKVKGDIIYGNECRNELLKGILTVSDVVKLTGP	99	

PILMAJOR	KGRNVILEMPYACPKITKDGVTVAKSIEFEDSFENLGANLVRQVAGLTNDNA---GOGTTTATVLSGAI FKEGFRSVASGTHPNHDLKRGIDOLACREVLIS	143
PITCRUZ	KGRNVII EQSYGAPKITKDGVTVAKAIEFKDPFENMGAQLVRQVCKNTNDLA---GOGTTTSAVLVASVFSSESLRCIATGTNPIDMKRGMDRAVGVILQS	137
P1PROHU	KGRTVII EQSWGSPKVTKDGVTVAKSIDLKDKYKNI GAKLVQDVANNTNEEA---GOGTTTATVLARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	154
P1MOUSE	KGRTVII EQSWGSPKVTKDGVTVAKSIDLKDKYKNI GAKLVQDVANNTNEEA---GOGTTTATVLARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	154
P1RAT	KGRTVII EQSWGSPKVTKDGVTVAKSIDLKDKYKNI GAKLVQDVANNTNEEA---GOGTTTATVLARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	154
P1CHO	KGRTVII EQSWGSPKVTKDGVTVAKSIDLKDKYKNI GAKLVQDVANNTNEEA---GOGTTTATVLARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	154
P1MOTH	KGVNVI LAKNLGPPKITKDGVTVAKGIDLKDLFQNI GARLVQNVANNTNEEA---GOGTTTATVLARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	157
P1CUCURB	KGRTVII EQSFGAPKVTKDGVTVAKSIEFKDKVKNVGSASLVQVANATNDVA---GOGTTTATVLTARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	161
ARATHMIT	KGRNVVIEQSWGAPKVTKDGVTVAKSIEFKDKVKNVGSASLVQVANATNDVA---GOGTTTATVLTARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	160
MAIZEMIT	KGRNVVIEQSWGAPKVTKDGVTVAKSIEFKDKVKNVGSASLVQVANATNDVA---GOGTTTATVLTARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	163
BNAPUSMT	KGRNVII EQSWGAPKVTKDGVTVAKSIEFKDKVKNVGSASLVQVANATNDVA---GOGTTTATVLTARSAKEGFEKISKGANPVEIRRGVMLAVDAVIAE	164
P1ECOLI	KGRNVVLDKSF GAPITKDGVTVAREIELEDKFENHGAQHVKEVASKANDAA---GOGTTTATVLAQAIIIEGLKAVAAGHNPMDLKRGIKAVTAAVEE	130
P1HLEP	KGRNVVLEKKWGAPITKDGVTVAREIELEDKFENHGAQHVKEVASKANDAA---GOGTTTATVLAQAIIIEGLKAVAAGHNPMDLKRGIKAVTAAVEE	129
P1PLAFA	RGRNVVLEKEYGSPLIINDGVTAKNISLDRKKNNGVKLMQESTNISMDKA---GOGTSTALMTATITKKGIEQVNRNHNPIIQRGILQASKMIIEK	196

PILMAJOR	LAEQSRPVTSKSEITQVAMISANMDEIGSLIGDAMQVVGKGVITTOEGRSLNTELELVEGMSFERGYTSPYFVTNTKAQRCELENALVYVANRKLTSV	243
PITCRUZ	VAEQNRKVTSTENIVQVATISANGDEELGRLIGAMEKVGKGVITTOGDKTMTTELEVVEGMSIDRGYISPYFVTDAAKQASWRMLVLVSACKVSSI	237
P1PROHU	LKKQSKPVTTPEEIAQVATISANGKEIGHIISDAMKKVGRKGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSI	254
P1MOUSE	LKKQSKPVTTPEEIAQVATISANGKDIGNIISDAMKKVGRKGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSV	254
P1RAT	LKKQSKPVTTPEEIAQVATISANGKDIGNIISDAMKKVGRKGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSV	254
P1CHO	LKKQSKPVTTPEEIAQVATISANGKDIGNIISDAMKKVGRKGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSV	254
P1MOTH	LKEMSKPVNTSEEIAQVATISANGDESIGKLI AAMNRVGNVGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSV	257
P1CUCURB	LKSRARMISTSEEIAQVATISANGEREIGELIAKAMEKVGKEGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSI	261
ARATHMIT	LKSKARMISTSEEIAQVATISANGEREIGELIAKAMEKVGKEGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSI	260
MAIZEMIT	LKGHARMISTSEEIAQVATISANGEREIGELIAKAMEKVGKEGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSI	263
BNAPUSMT	LKSRARMISTSEEIAQVATISANGEREIGELIAKAMEKVGKEGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSI	263
P1ECOLI	LKALSVPSCDSKATIAQVATISATSDETVGLKIAEAMDKVGKEGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSI	230
P1HLEP	LLDKAEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGKEGVITVKGDKTLNDELEIEGKMFDRGYISPYFINTSKGQKCEFODAYVLLSEKKISSI	228
P1PLAFA	IKSLSTPIKTYDILNIAATIASNNDVHNGQIIANAYDKLGKNAIILDONADINDKLEFTEGYNFORGIINPYLYNENKDYIEYSNVSTLITDQNDINI	296

PILMAJOR	AHILPALNYAIQOKRPLLVIAEDVEGEAMHTFLYNKIQRISGCAVKAPGFGDMRINQLODIAVFTGSQMI SED-LGLSLDQND FSERFLGTCRKVTYSR	342
PITCRUZ	HTILPALNHVVGTRPLLIADOVESEALTTMI FNKLQGLKLIACVKAPGFGDNKTAHMDIAI FAGARLVGEEGSGLEDAENFOPAILGTVYKATITK	337
P1PROHU	QSI VPALEIA NAHRKPLVIAEDVGEALSTLVNLKVLQVAVKAPGFGDNKRNKQLDMAIATGGAVFGEGLNLNLE--DVQANDLGKVGVEIVTK	352
P1MOUSE	QSI VPALEIA NAHRKPLVIAEDVGEALSTLVNLKVLQVAVKAPGFGDNKRNKQLDMAIATGGAVFGEGLNLNLE--DVQANDLGKVGVEIVTK	352
P1RAT	QSI VPALEIA NAHRKPLVIAEDVGEALSTLVNLKVLQVAVKAPGFGDNKRNKQLDMAIATGGAVFGEGLNLNLE--DVQANDLGKVGVEIVTK	352
P1CHO	QSI VPALEIA NAHRKPLVIAEDVGEALSTLVNLKVLQVAVKAPGFGDNKRNKQLDMAIATGGAVFGEGLNLNLE--DVQANDLGKVGVEIVTK	352
P1MOTH	SOVVPAL ELANSOKKPLVIAEDYDGEPLSVLVNKLKIGLPVAVKAPGFGGEYRTHALLDMAAATGG-VFEDOTNLVRL--DCQAESFGOVGEIVITK	354
P1CUCURB	NAVVKYLELAKKORPLIIVSEDVESEALATLILNKL RAGIKVCAIKAPGFGENRKAGLOLAVLTGGQVITEE-LGNWLE--KVDLMLGSCCKKITISK	358
ARATHMIT	NSIVKYLELAKKORPLIIVSEDVESEALATLILNKL RAGIKVCAIKAPGFGENRKAGLOLAVLTGGQVITEE-LGNWLE--KVDLMLGSCCKKITISK	357
MAIZEMIT	NAVVKYLELAKKORPLIIVSEDVESEALATLILNKL RAGIKVCAIKAPGFGENRKAGLOLAVLTGGQVITEE-LGNWLE--KVDLMLGSCCKKITISK	360
BNAPUSMT	NSIVKYLELAKKORPLIIVSEDVESEALATLILNKL RAGIKVCAIKAPGFGENRKAGLOLAVLTGGQVITEE-LGNWLE--KVDLMLGSCCKKITISK	360
P1ECOLI	REMLPVLEAVAKAGPLLIADVEGEALATAVVNTIRGIVKAAVKAPGFGDRRKAMLODIATLTGGTVISEE-IGMELEKATLE--DLGQAKRVVINK	327
P1HLEP	KDILLPLEKIVQAGKSLIIAEDVEGEALSTLVNKLIRGTFKSVAVKAPGFGDRRKAMLODIATLTGGTVISEE-IGMELEKATLE--DLGQAKRVVINK	325
P1PLAFA	QSILPILEIAFKNKQPLCIADDFSNVLTQTLINLKGAKIVLCIVTNSKYISADVGLDNLNHNHNSFDNNYLSL-----LGSANTLIVCK	385



P1LMAJOR	-----LESISKDRRLPPDIRTGVNI VKKATGLPARYIANNAGVEGSV VAGKVLARKDP-SFGYNAQTGEYVNMFEAGIIDP	515
P1TRCRUZ	-----LDSSLGSSSLTADQRTGVQIIPNAVRLPAHTIVLNAGKEGAVVYKVLNNQDV-TVGYDAQRDRYVNMFEAGIIDP	510
P1PROHU	-----LDLSLPANE---DQKIGIEIKRTLKIPAMTIAKNAGVEGSLIVEKIQSS-S-EVGYDAMAGDFVNMVEKGIIDP	521
P1MOUSE	-----LDLSKPANE---DQKIGIEIKRALKIPAMTIAKNAGVEGSLIVEKIQSS-S-EVGYDAMLGDFVNMVEKGYIDP	521
P1RAT	-----LDLSKPANE---DQKIGIEIKRALKIPAMTIAKNAGVEGSLIVEKIQSS-S-EVGYDAMLGDFVNMVEKGIIDP	521
P1CHO	-----LDLSKPSNE---DQKIGIEIKRALKIPAMTIAKNAGVEGSLIVEKIQSS-S-EIGYDAMLGDFVNMVEKGIIDP	521
P1MOTH	-----LDLLKPANK---DQKIGVSIKKALRTPCITIASNAGFDGAVVSKVEQMD-P-EYGYDALMNEYVNMIEKGIIDP	523
P1CUCURB	-----LDKLPATANF---DQKIGVQIQNALKTPVHTIASNAGVEGAVVVGKLLLEQDDP-DLGYDAAKGEYVDMVKAGIIDP	528
ARATHMIT	-----LEKLPATANF---DQKIGVQIQNALKTPVYTIASNAGVEGAVIVGKLLLEQDNP-DLGYDAAKGEYVDMVKAGIIDP	527
MAIZEMIT	-----LDKLPATANF---DQKIGVQIQNALKTPVHTIASNAGVEGAVVVGKLLLEQNT-DLGYDAAKGEYVDMVKGTIIDP	530
BNAPUSMT	-----LDKLPATANF---DHKIGVQIQNALKTPVYTIASNAGVEGAVIVGKLLSDNP-DLGYDAAKGEYVDMVKSGIIDP	531
P1ECOLI	-----LADLRGONE---DQNVGIKVALRAMEAPLRQIVLNCGEEPSVYANTY-KGGDG-NYGYNAATTEYGNMIDMGIIDP	496
P1HLEP	-----LDLKL-LTG---DEATGANIVKVALEAPLKQIAFNSGMEPGVYAEKV-RNLVS-HGLNNAATGEYDOLLKAGVADP	493
P1PLAFA	F1QEIHKKIIEEDLQISSNDEKKYLELGNLSEMEELQMGANIVSSLDVITKQIADNAGNVGNMYXKILNSDKYGFQDYDNTNKFVNMVEKGIIDS	584

P1LMAJOR	MKVYKSAVVNACSVAGHMITTEAAVVEKDLLGREKRIEDEGMEDKEKKRSVDKLRKQVNEAGRTOAQOGAADEV	586
P1TRCRUZ	ARVVRVAITDPVSVASLMTTEASIVD---FPKEETPAAGGMGGMGGMGGMDMY-----	562
P1PROMU	TKVVRTALLDAAGVASLLTTAEVVTE---IPKEEKDPGM-----GAMGGMGGMGG-GMF-----	573
P1MOUSE	TKVVRTALLDAAGVASLLTTAEVVTE---IPKEEKDPGM-----GAMGGMGGMGG-GMF-----	573
P1RAT	TKVVRTALLDAAGVAPLLTTAEVVTE---IPKEEKDPGM-----GAMGGMGGMGG-GMF-----	573
P1CHO	TKVVRTALLDAAGVASLLTTAEVVTE---IPKEEKDPGM-----GAMGGMGGMGG-GMF-----	573
P1MOTH	TKVVRRLTDSAGVASLLTTAEAVICD---MPKQ-KDPPP---EY-----APIGG-GDY-----	569
P1CUCURB	LKVIRTALVDAASVSSLMTTTEVVVE---LPKDENEVPAMGGG-----MGG-M---DY-----	575
ARATHMIT	LKVIRTALVDAASVSSLTTTEAVVVD---LPKDESESGAAGGG-----MGG-MVV-MDY-----	577
MAIZENIT	LKVIRTALVDAASVSSLMTTTEIIVE---IPKEEAPAPAMGGG-----MGG-M---DY-----	577
BNAPUSMT	VKVIRTALVDAASVSSLTTTEAVVTE---IPTKEDASPMAGGGGGGGMGGMGG-MGG-MGF-----	587
P1ECOLI	TKVTRSALQYAAASVAGLMTTTECHYVD---LPKNDAAIIGAAGMGGMGGMGGMGMH-----	548
P1HLEP	VKVTRSALQYAAASVAGLFLTTEAVVD---KPEKTA--PASDPTGGMGGMDF-----	541
P1LAFAL	INVIISVINKSCSIASHVLTTECHYVDEHKDKGLDSSINSPNYLSKHRRYTKHKLHDDDEDDEDEEDEDDE	658

The asterisks (\*) and periods (.) below the sequence alignment indicate, respectively, residues that are identical or similar in these species. Phylogenetic analysis was carried out on the region beginning with the 5' sequence PKDIRYGM and ending with 3' sequence TTEAAVVE (between residues 15-542) which could be aligned without ambiguity in all homologs. This alignment highlights the distinct carboxy-terminus (amino acids 542-589) of the leishmania *HSP60* gene as compared with other HSP60 homologs. P1LMAJOR=HSP60 from *L. major*, P1TCRUZI= HSP60 from *T. cruzi*, P1PROHU=HSP60 from human, P1MOUSE= murine HSP60, P1RAT=rat HSP60, P1CHO=hamster HSP60, P1MOTH=HSP60 from *Heliothis virescens*, P1CUCURB=HSP60 from Cucurbita, ARATHMITH=HSP60 from *Arabidopsis thaliana*, MAIZEMIT=HSP60 from maize, BNAPUSMT=*Brasica napus* HSP60, P1ECOLI=*E. coli* GroEL, P1MLEP=*M. leprae* HSP60, and P1PLAFA=HSP60 from *P. falciparum*.

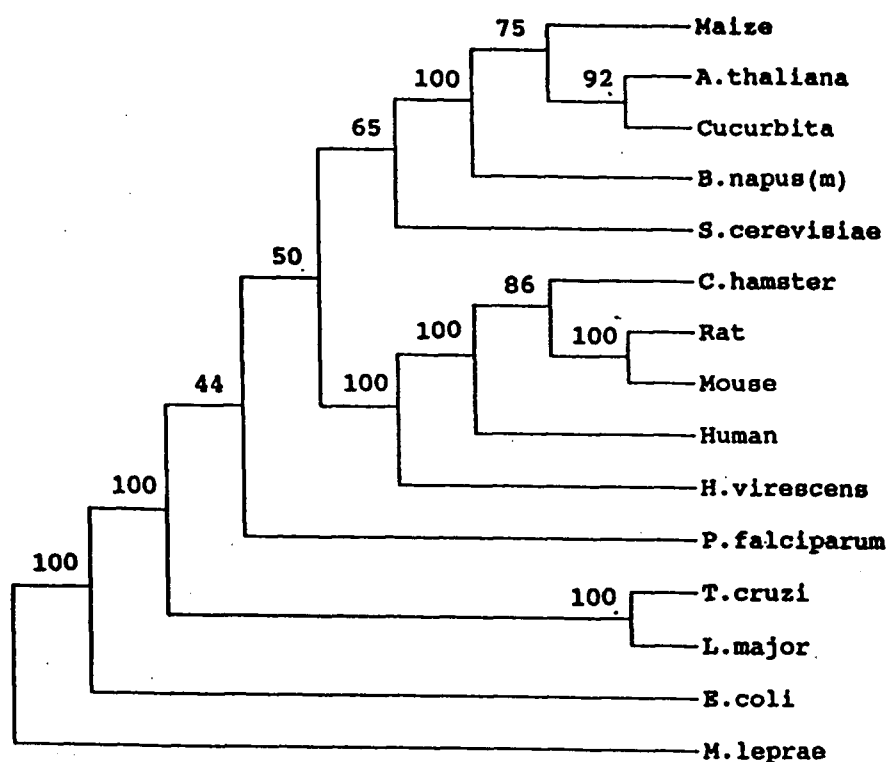
A pairwise similarity matrix of HSP60 sequences is shown in Table 3. The *L. major* HSP60 shows maximum identity/similarity (54.6% identical amino acids plus additional 15% conservative substitutions) to the *T. cruzi* homolog. Less extensive amino acid sequence similarity (48%) is observed between *L. major* HSP60 and the corresponding human protein (293). Notably, significant regions of sequence dissimilarity between the *L. major* and human HSP60 are identified and these are concentrated principally within the carboxy-terminal regions of the proteins. In comparison to other eukaryotic/prokaryotic putative HSP60 polypeptides, the homolog from *P. falciparum* is highly divergent (length of about 700 a.a. as compared to lengths ranging between 540-580 for others) and shows low sequence similarity to all other homologs.

Table3. Amino Acid Identity/Similarity among HSP60 Sequences

	A	B	C	D	E	F	G	H	I	J
A. <i>L. major</i>		46.8	46.9	54.6	33.4	44.1	48.6	48.0	47.1	49.2
B. Human	65.3		97.4	51.8	35.3	64.1	56.1	57.8	50.5	46.8
C. Mouse	65.4	98.4		51.6	35.1	64.5	55.4	57.8	50.4	47.7
D. <i>T. cruzi</i>	69.7	68.5	68.5		33.3	48.2	52.8	55.7	49.6	47.9
E. <i>P. falciparum</i>	52.0	55.7	55.5	50.9		34.4	35.7	35.7	34.3	33.6
F. <i>H. virescens</i>	59.4	75.9	76.3	61.7	53.0		50.4	51.7	47.4	44.2
G. <i>S. cerevisiae</i>	64.5	72.4	71.7	69.5	55.6	68.1		56.6	53.8	47.9
H. Maize (m)	65.2	74.4	74.2	71.7	53.7	66.6	72.3		55.1	52.1
I. <i>E. coli</i>	62.6	68.4	68.5	66.2	55.8	62.2	68.9	68.6		59.2
J. <i>M. leprae</i>	64.4	64.4	64.5	64.9	51.2	58.2	65.3	68.4	72.2	

The amino acid identity (upper triangle) or similarity (includes identical plus conservative amino acid replacements; lower triangle) between pairs of sequences was determined as described in Materials and Methods.

The amino acid sequence alignment of diverse HSP60 proteins shown in Figure 21 was used to construct a phylogenetic tree (Figure 22). Results indicate that HSP60 of *L. major* branches 100% of the time with the homolog from *T. cruzi* indicating a close evolutionary relationship between HSP60s from these two organisms. The HSP60 homologs from animal and plant species form separate groups with high affinities between members. Furthermore, as expected within the eukaryotic lineage, these protist species show much earlier branching than do either the animal, plant or fungal species. These results are similar to those observed in earlier studies (294).



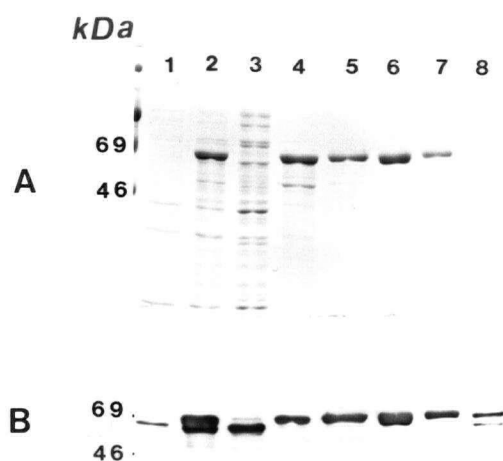
**Figure 22. Evolutionary relationship between the eukaryotic species based on HSP60 sequences.**

The tree shown here is a neighbor-joining (295), consensus tree obtained after 100 bootstraps (296). The numbers at the branch points indicate the % of time the species to the right grouped together and it provides a measure of the reliability of their branching order in the tree.

## 2. Expression and purification of rLHSP60.

To enhance expression of recombinant *HSP60* in *E. coli*, the leishmania *HSP60* gene was modified and then amplified by PCR [as described previously (271)] using IEMBL-3 clone 232 DNA as template. As shown in Figure 19, codons in the vicinity of the ATG start codon were changed to match *E. coli* codon usage without altering the ultimate amino acid sequence as described for high level expression of gp63 (271). Thus, the following codon changes were made: CTC to CTT, TCC to TCT, ACG to ACT and CGC to CGT which code respectively for leucine, serine, threonine and arginine. The modified gene was cloned into the pET-3a expression vector and this resulted in high level expression of rLHSP60 representing approximately 50% of total bacterial protein (Figure 23A, lane 2).

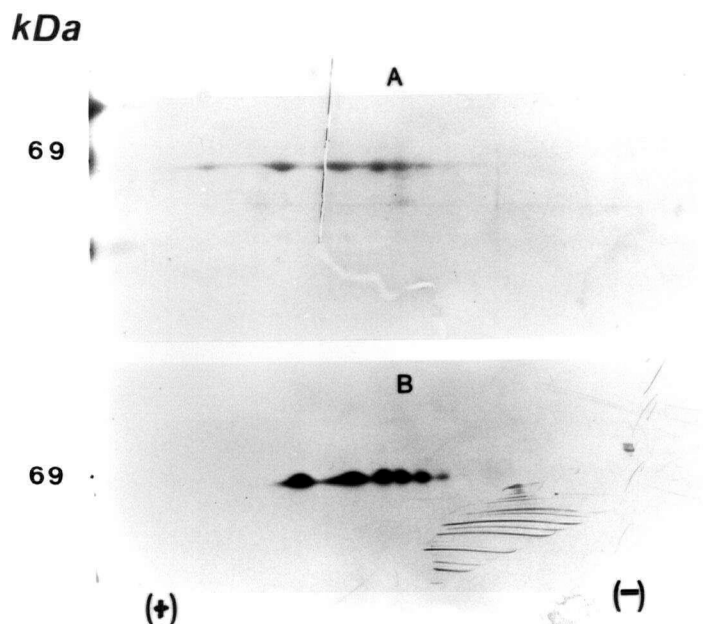
To purify rLHSP60 from *E. coli*, clone pET-LHSP60 (containing the modified leishmania *HSP60* gene) was grown in 250 ml LB medium, cells were lysed and inclusion bodies containing rLHSP60 were isolated as described (271). SDS-PAGE analysis of the different fractions revealed that most *E. coli* protein remained in the supernatant while the rLHSP60 co-purified with the inclusion bodies (Figure 23, panel A, lanes 3 and 4, respectively). Proteins within the inclusion bodies were purified further by three rounds of separation on and elution from SDS-PAGE gels (Figure 23, panel A, lanes 5, 6 and 7, respectively). Recovery of protein was ~25 mg per liter of culture.



**Figure 23. Expression and purification of *L. major* HSP60 as analyzed by SDS-PAGE and detected by Coomassie blue staining (A) or by immunoblotting (B).**

Aliquots of growing pET-LHSP60-containing *E. coli* were taken before (lane 1) or after IPTG induction (lane 2). Inclusion bodies (IB) (lane 4) containing the rLHSP60 were separated from the bulk of *E. coli* protein (lane 3) by centrifugation. HSP60 was purified from IB's by 3 rounds (lanes 5, 6 and 7) of purification from SDS-PAGE gels. Recombinant mycobacterial HSP65 is shown in lane 8 for reference. Molecular size markers are shown on the left.

Immunoblotting using anti-HSP60 polyclonal antibody from *Cyanobacteria* was used to monitor the purification of rLHSP60 and to distinguish it from *E. coli* GroEL, the HSP60 homolog in *E. coli*. As shown in Figure 23B, while only GroEL ( $M_r \sim 60,000$ ) is expressed in uninduced cells (lane 1), both GroEL and rLHSP60 ( $M_r \sim 65,000$ ) are present in the IPTG-induced pET-LHSP60 clone (Figure 23B, lane 2). While GroEL remains in the supernatant (Figure 23B, lane 3) rLHSP60 purifies with the inclusion bodies (panel B, lane 4). To confirm the absence of contaminating proteins, purified rLHSP60 was analyzed by 2-dimensional SDS-PAGE. Six spots all with approximate sizes of  $M_r 65,000$  and with isoelectric points between 6.0-7.0 were detected by silver staining (Figure 24A) panel A). Apparently identical spots were also detected by immunoblotting with the anti-HSP60 polyclonal antibody raised against *Cyanobacteria* (Figure 24B).



**Figure 24. Two dimensional SDS-PAGE analysis of purified, rLHSP60 detected by silver staining (A) and by immunoblotting (B).** rLHSP60 was purified as described in the legend to Figure 7. The purified protein (0.5 ug) was solubilized in two-dimensional gel electrophoresis sample buffer and separated on pH 3-10 isoelectric focusing gels and then electrophoresed on a second dimension in 10% SDS-PAGE. Gels were either silver stained or probed using polyclonal antibodies against *Cyanobacteria* HSP60. Fluorographs of the gels are shown with the acid and basic ends respectively to the left (+) and right (-). Molecular size markers are shown on the left.

### 3. Discussion

Initial studies in this thesis demonstrated that two isoforms (65- and 67-kDa) of leishmania HSP60 are selectively expressed and induced in infected macrophages (297). High level expression of leishmania HSP60 isoforms in infected cells suggested the possibilities that they may be involved in pathogenesis, as targets of the immune response or both. Further study of these molecules required the cloning and expression of the *HSP60* gene of *Leishmania*. A *L. major* genomic DNA library was screened with a PCR-generated probe for HSP60. This led to the identification and isolation of a DNA fragment containing the complete coding sequence of a gene which showed extensive sequence homology to other members of the HSP60 chaperonin family that are localized in mitochondria (Figures 20 and 21). Restriction enzyme and Southern analysis of genomic DNA suggested that the leishmania *HSP60* gene is present in the genome as a single copy gene. Thus, when either leishmania genomic DNA (Figure 18) or  $\lambda$ EMBL-3 clone 232 (containing the leishmania HSP60 sequence) (Figure 17) are digested with the enzyme *Acc* I (which cuts once inside the HSP60 DNA probe), two fragments of 1.0 and 0.7 Kb are produced. When digestion is carried out with *Xho* I (which cuts twice inside the DNA probe), two fragments of 0.6 and 3.6 Kb are generated. Alternatively, when the DNA is cut with either *Bam* HI, *Sma* I, *Hind* III or *EcoR* I, all of which cut outside the probe, only single fragments are obtained of respectively 9.0, 5.0, 23.0 and 23.0 Kb. This pattern is consistent with the presence of a single copy gene.

Presently, the subcellular localization of the leishmania HSP60 is not known. However, the N-terminal amino acids 1-15 of LHSP60 represent an apparent mitochondrial targeting sequence (Figure 20). It is rich in basic (lysine and arginine) and hydroxyl-group containing (serine and threonine) amino acids and it is devoid of acidic residues, three features known to be characteristic of a mitochondrial localization sequence (292,298). In this respect, leishmania HSP60 is similar to HSP60 from *T. cruzi* (292), *H. virescens* (299), human (293) and hamster (300) all of which contain mitochondrial targeting sequences. On the other hand, this feature distinguishes leishmania HSP60 from GroEL of *E. coli* and the *M. leprae* HSP60 (Figure 21) both of which lack such a sequence.

HSP60 from various species have been shown to be highly immunogenic molecules for both B and T cells (46,301). Immunization of mice with HSP60 from either mycobacteria (302) or yersinia (164) has been shown to induce protective immunity in murine models of tuberculosis and yersiniosis. Moreover, guinea pigs immunized with HSP60 from *L. pneumophila* are also protected against lethal challenge with this organism (167). The findings of the present study demonstrate significant regions of sequence dissimilarity between *L. major* HSP60 and its human homolog, particularly in the C-terminal region. Taken together with the clear vaccine potential of HSP60 that has been shown for other intracellular pathogens, this suggests that it should be possible to select peptide sequences within LHSP60 with the potential to engender protective immunity and which are also unlikely to provoke autoimmune responses. Furthermore, the findings that HSP60 from diverse intracellular pathogens can protect animals against subsequent infection, raises the possibility that rLHSP60 may also be involved in protection against infection with *Leishmania*.

### **C. IMMUNOLOGICAL PROPERTIES OF LHSP60**

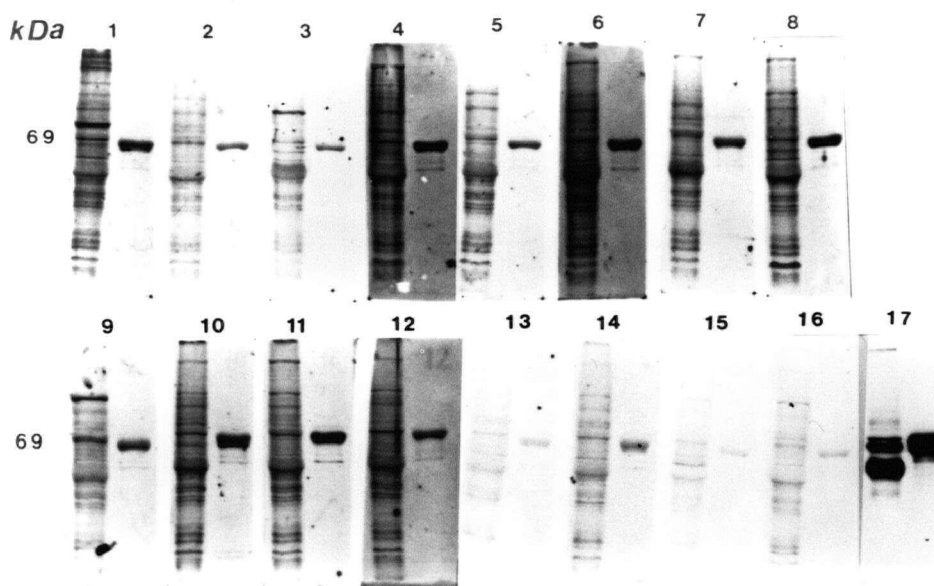
To examine immunological properties of LHSP60 and to test the hypothesis that HSP60 from *Leishmania* may confer protection against infection, two types of experiments were done using purified rLHSP60 as antigen. First, the question of whether HSP60 is recognized during infection was addressed by testing sera from patients with American tegumentary leishmaniasis for the presence of anti-HSP60 antibodies. Second, the ability of this protein to induce protection against leishmania infection in immunized Balb/c mice was studied.

#### **1. LHSP60 is recognized by sera from patients with American tegumentary leishmaniasis**

Purified rLHSP60 was used to determine whether sera from patients with active American cutaneous leishmaniasis, from an endemic zone in the southwestern region of Colombia contained antibodies to LHSP60. Sera from healthy North American, non-exposed donors were used as negative controls. Immunoblotting showed that sera from patients with

leishmaniasis reacted with significantly greater intensity with both total leishmania lysate and also with rLHSP60 (Figure 25).

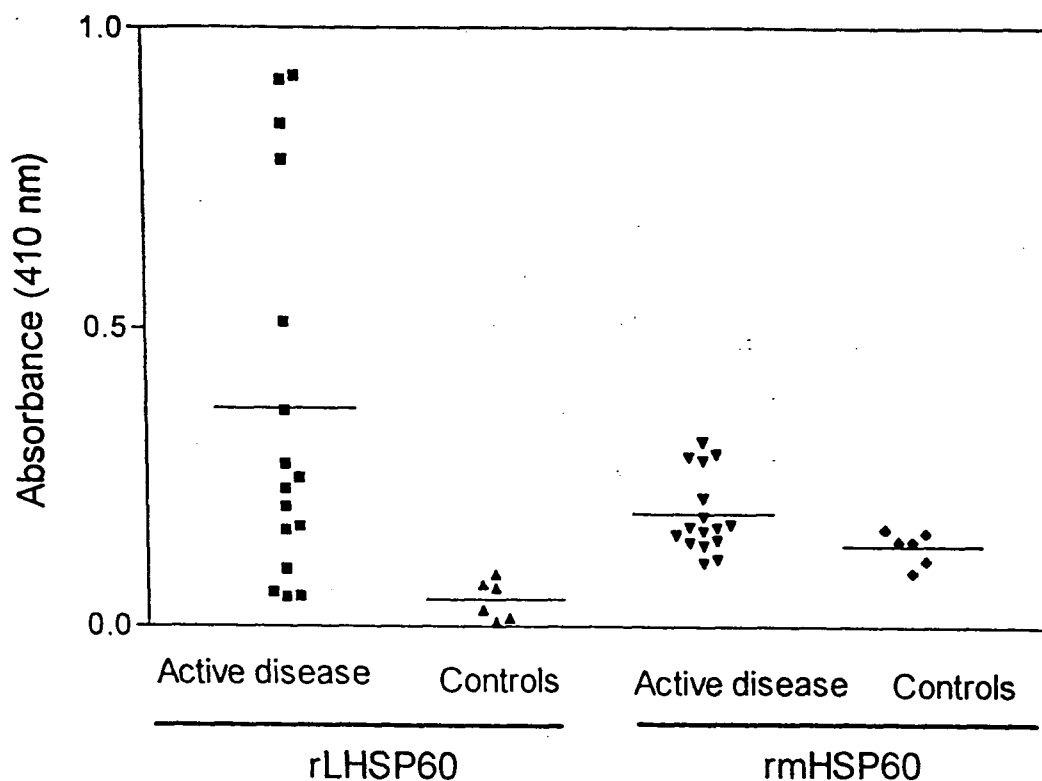
When measured by ELISA (Figure 26), reactivity of patient sera with rLHSP60 was  $0.37 \pm 0.32$  (optical density  $\pm$  SD) and this was significantly higher ( $P=0.0001$  by unpaired  $t$  test) than that ( $0.04 \pm 0.03$ ) of control sera. Furthermore, also as shown in Figure 26, this reactivity appeared to be specific, since sera from patients with American cutaneous leishmaniasis reacted with recombinant mycobacterial HSP60 (rMHSP60) to the same extent as did sera from healthy donors (optical density  $\pm$  SD =  $0.18 \pm 0.06$  for patients vs  $0.14 \pm 0.03$  for controls).



**Figure 25. Immunoblotting of purified, *L. major* rHSP60 using sera from patients with American cutaneous leishmaniasis and normal subjects.**

20  $\mu$ g of protein from either *L. major* promastigotes or 0.4  $\mu$ g of rLHSP60 were separated by 10% SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes and then analyzed by immunoblotting using sera from patients with leishmaniasis (panels 1-12) or normal, unexposed controls from the Red Cross (panels 13-16). In panel 17 polyclonal antibody against *Cyanobacteria* HSP60 was used. For each panel, the left lane corresponds to total leishmania lysate and right lane to rLHSP60. Exposure conditions were the same for all blots. Molecular size markers are shown on the left.





**Figure 26.** Quantitative analysis by ELISA of antibodies to HSP60 in sera from patients with American cutaneous leishmaniasis and in sera from healthy donors. Serum samples from 16 patients collected in an endemic zone in Colombia and sera from 6 healthy, non-exposed subjects were analyzed using either recombinant leishmania (rLHSP60) or recombinant mycobacterial HSP65 (rmHSP65) as antigens. Wells were coated with antigen at 5 µg/ml and sera were used at a 1:4000 dilution.

## 2. Immunization of Balb/c mice with rLHSP60

Recent investigations demonstrated that vaccination of Balb/c mice with a soluble leishmania antigen (SLA) in the presence of IL-12 as adjuvant promoted the development of CD4<sup>+</sup> Th1 cells. These mice were resistant to subsequent infection with *L. major* (213). Similarly, a 36 kDa leishmania antigen (LACK) of the tryptophan-aspartic acid repeat family was found to be protective when administered to Balb/c mice together with IL-12 before infection (241). These experiments demonstrated that IL-12 may be an effective adjuvant with

the potential to replace conventional adjuvants used for vaccination. These findings, together with the protective potential of HSP60 in other systems of intracellular infection, suggested that study of rLHSP60 together with IL-12 was warranted.

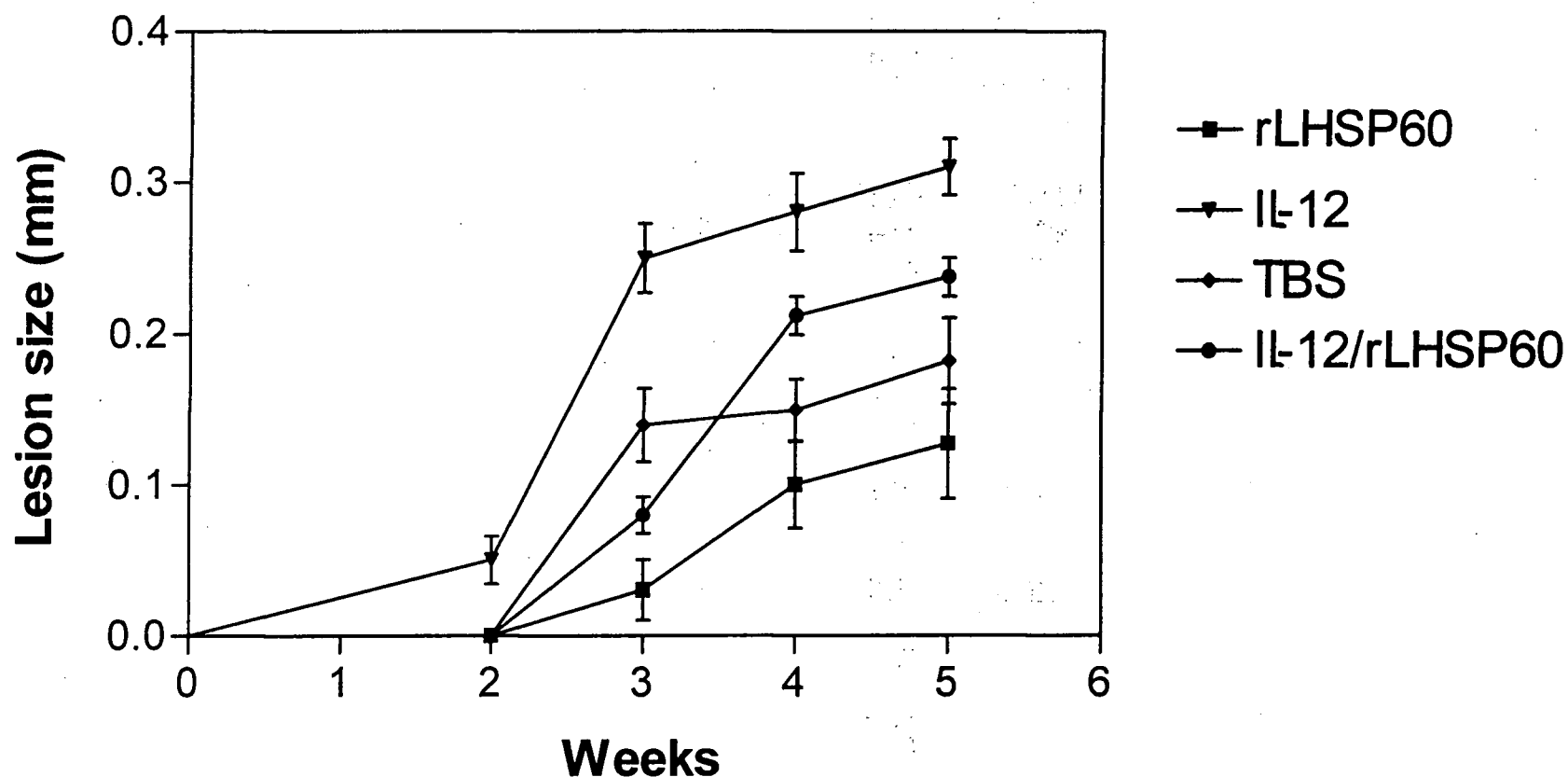
To examine the vaccine potential of this combination, groups of Balb/c mice were immunized three times with either rLHSP60 alone, rLHSP60 plus IL-12, IL-12 alone or TBS. Animals were then infected in the footpad with promastigotes of *L. major*. The course of infection was monitored by measuring footpad swelling at weekly intervals using an engineer's caliper. Mice were then sacrificed and numbers of parasites in lesions and proliferation of lymph node cells in response to rLHSP60 were assessed.

Figure 27 shows the course of lesion development in immunized mice and illustrates that rLHSP60 alone significantly protected mice during the first four weeks after leishmania infection. For data analysis, measurements of lesion size from weeks 2-4 were pooled (Figure 28) and analyzed by ANOVA. Overall, this showed that significant differences were present with  $P=0.0001$ . In respect to individual comparisons, mean lesion size in mice immunized with rLHSP60 alone was  $0.08 \pm 0.02$  mm (mean  $\pm$  SEM) as compared to: (i)  $0.3 \pm 0.02$  mm in mice given TBS alone [ $P=0.01$ , by Tukey-Kramer multiple comparison test (T-KMCT)], (ii) mice immunized with either IL-12 alone ( $0.6 \pm 0.05$  mm) ( $P=0.001$ , by T-KMCT) or to recipients of rLHSP60 plus IL-12 ( $0.3 \pm 0.02$  mm) ( $P=0.01$ , by T-KMCT). Surprisingly, mice treated with IL-12 alone developed the largest lesion size of all and this difference persisted at least until five weeks post-infection (Figure 27). However, by week seven this pattern changed significantly. TBS-immunized animals presented the largest lesion sizes ( $0.95 \pm 0.13$ ) while the IL-12 immunized group showed the smallest lesion size of all ( $0.66 \pm 0.32$ ) (Figure 29). Animals immunized with rLHSP60 alone or with rLHSP60 plus IL-12 showed lesions of  $0.81 \pm 0.26$  and  $0.83 \pm 0.11$ , respectively (Figure 29).

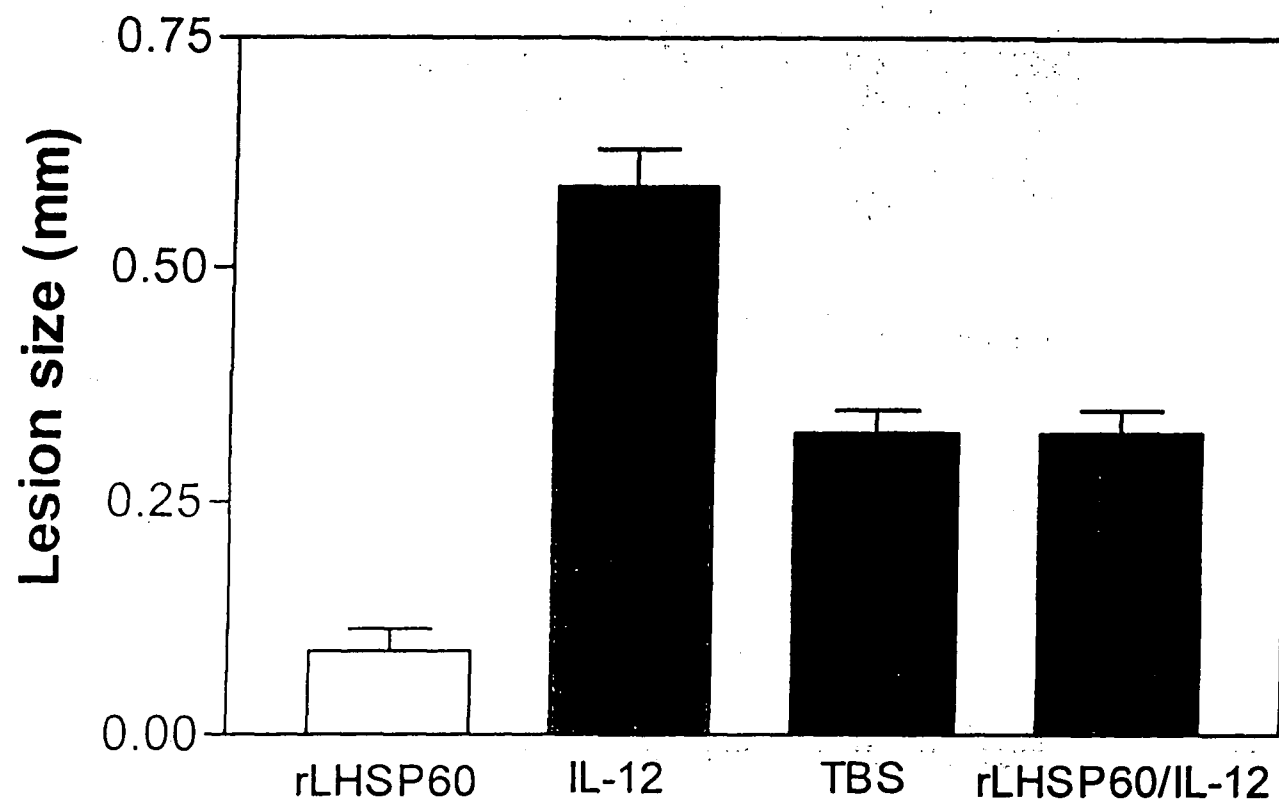
Parasite loads in lesions of immunized animals were determined seven weeks post-infection. Results show that parasite burdens in tissues of different immunized groups correlated with lesion size (Figure 30). Thus, mice immunized with either IL-12 or rLHSP60 plus IL-12 which showed the smallest lesions, also had the lowest parasite loads ( $4.9 \pm 1.1$  and

$4.8 \pm 1.2$ , respectively, mean  $\pm$  SD of log<sub>10</sub> total organisms per lesion). In contrast, control mice or mice immunized with rLHSP60 alone, which had larger lesions at week seven, had correspondingly larger numbers of parasites in tissue ( $12 \pm 0.8$  and  $9.3 \pm 4.3$ , respectively). These data show that immunization with rLHSP60 leads to reduced lesion size early after infection, but does not confer protection beyond ( $>$  five weeks). The results also demonstrate that combining rLHSP60 with IL-12 did not improve upon the results seen with IL-12 alone and appear to confirm results obtained by others indicating that IL-12 alone is able to confer some degree of protection against leishmania infection (199,212,213).

To determine whether LHSP60-specific T cells developed in the lymphoid organs of HSP60 immunized mice, spleen and popliteal lymph node cells were isolated seven weeks after infection and tested in a *in vitro* proliferation assay using rLHSP60 as antigen. As compared to spleen cells from TBS or IL-12 immunized mice, spleen cells from rLHSP60 or rLHSP60/IL-12 immunized mice proliferated modestly in response to rLHSP60 (Figure 31). In comparison to spleen cells, popliteal lymph node cells of rLHSP60 or rLHSP60/IL-12 immunized animals proliferated to a somewhat greater extent in response to rLHSP60 (Figure 32). These responses were significantly greater than those of lymph node cells from control (TBS) or IL-12 immunized mice ( $P=0.001$ , by ANOVA). Thus, although HSP60-responsive T cells appear to be present in lymph nodes of LHSP60 immunized mice, these cells apparently provide only limited protection under the conditions of this experimental system.

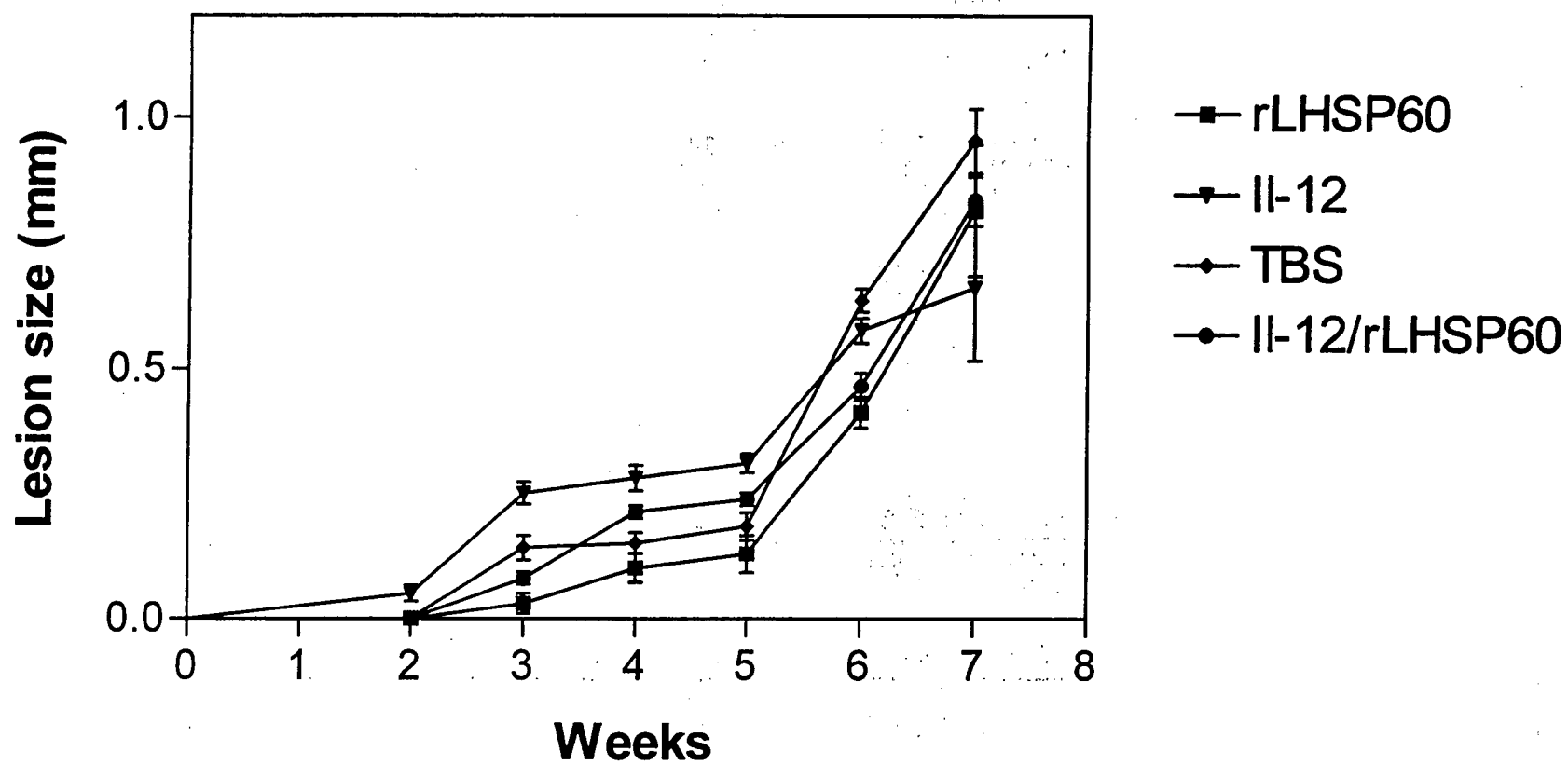


**Figure 27. Early protective effect of rLHSP60 against *L. major* infection in Balb/c mice.** Mice were vaccinated intracutaneously (IC) with either TBS, IL-12 (1  $\mu$ g), rLHSP60 (25  $\mu$ g) or a combination of IL-12 plus rLHSP60 (1 and 25  $\mu$ g respectively). This immunization protocol was repeated 10 and 25 days later and mice were then challenged in the right footpad with  $2 \times 10^6$  stationary phase *L. major* promastigotes. Lesion development was measured at weekly intervals using a caliper. Statistical analysis of pooled data is shown in Figure 28. Each data point represents the mean  $\pm$  SEM of swelling observed in the footpads of 5 mice.



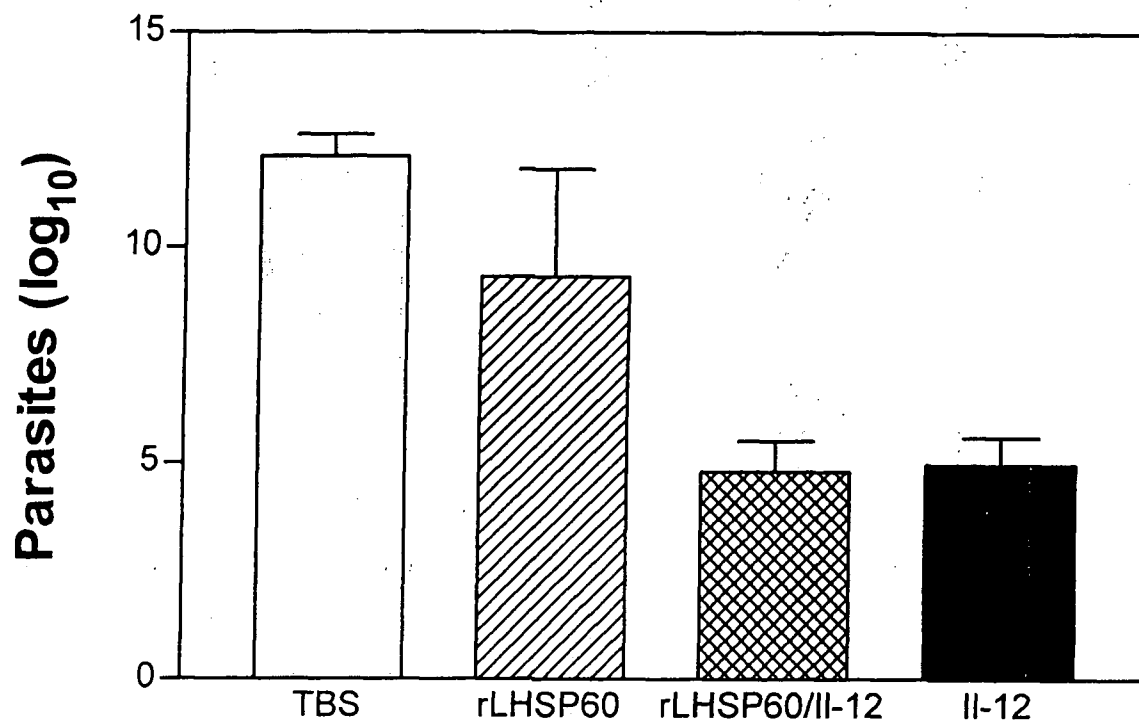
**Figure 28. Lesion sizes in mice immunized with rLHSP60 determined four weeks after infection.**

The remainder of the legend is as for Figure 27. Data (weeks 2-4) from Figure 27 were pooled and analyzed by ANOVA. This showed that significant differences were present ( $P=0.0001$ ). Individual comparisons of groups showed: rLHSP60 versus IL-12 ( $P=0.001$ ), rLHSP60 versus TBS ( $P=0.01$ ), rLHSP60 versus rLHSP60 plus IL-12 ( $P=0.01$ ), and IL-12 versus TBS ( $P=0.001$ ) as determined by (T-KMCT).



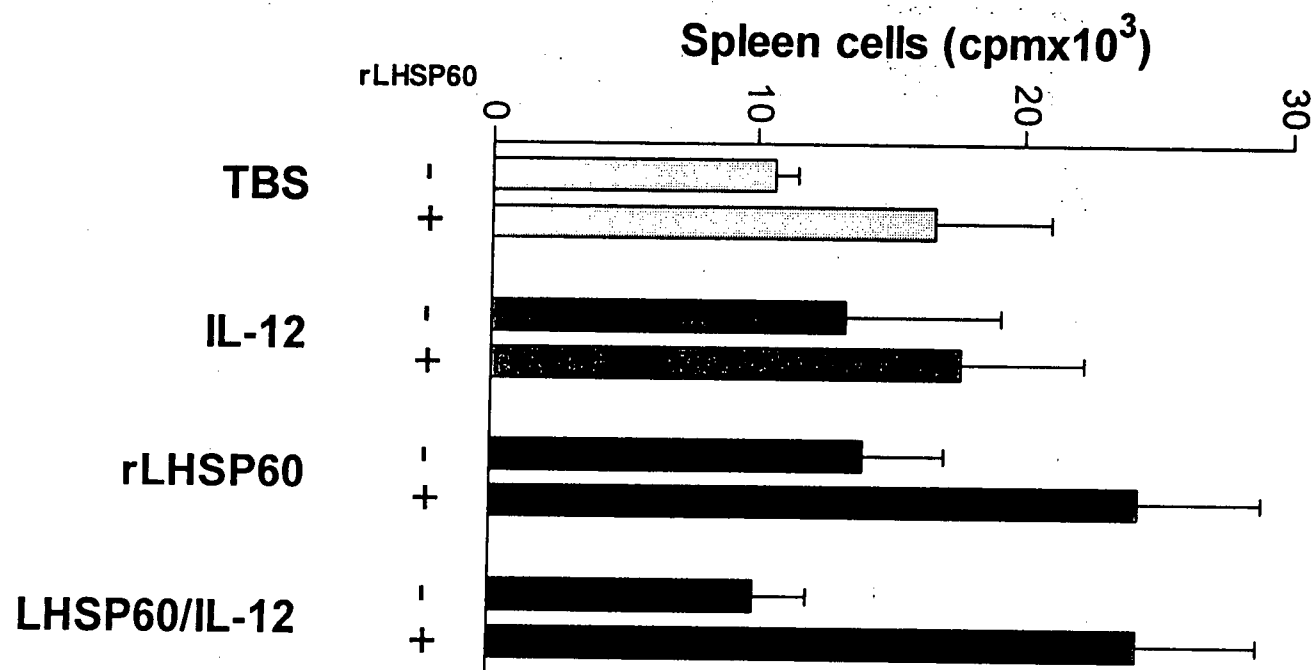
**Figure 29. Late lesion development of mice immunized with rLHSP60.**

The remainder of the legend is as for Figure 27. No statistical differences between groups were obtained 7 weeks after infection.



**Figure 30. Parasite loads in lesions of mice immunized with rLHSP60.**

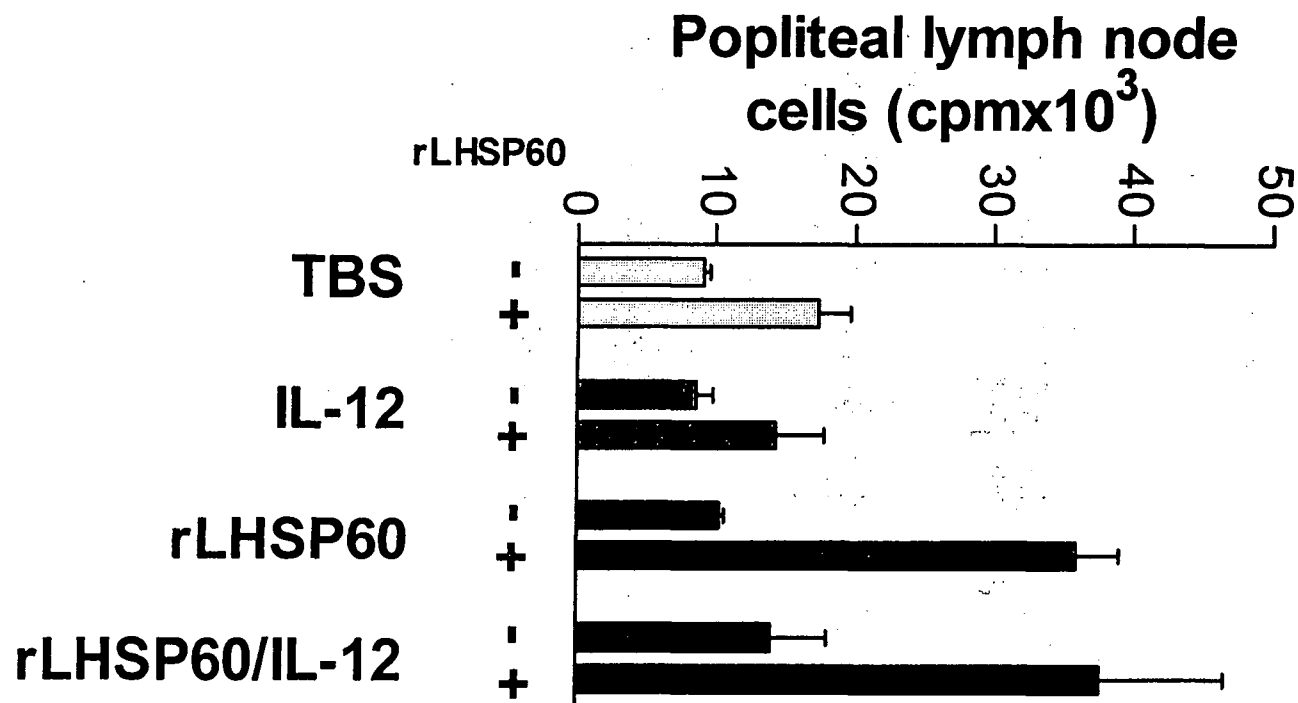
Mice were immunized as indicated in the legend to Figure 27 and then challenged with  $2 \times 10^6$  stationary phase *L. major* promastigotes. Seven weeks later mice were sacrificed and infected footpads were removed for isolation of leishmania from infected tissue. Quantitation of total parasite numbers in tissue was carried out by limiting dilution assay (LDA). Data represent the mean  $\pm$  SEM of values obtained from 5 animals per group. Analysis by ANOVA and multiple comparisons of groups showed: TBS vs rLHSP60 and IL-12 ( $P < 0.05$ ), TBS vs IL-12 ( $P < 0.05$ ), and TBS vs rLHSP60 ( $P > 0.05$ ).



**Figure 31. *In vitro* proliferation of spleen cells from rLHSP60 immunized mice.**

Balb/c mice were treated as indicated in the legend to Figure 27 and then challenged with  $2 \times 10^6$  stationary phase *L. major* promastigotes. Seven weeks later mice were sacrificed and spleen cells were isolated and cultured for 72 hr in the presence (+) or absence (-) of rLHSP60 (10  $\mu$ g/ml). Cells were incubated with [<sup>3</sup>H]-thymidine for the final 18 hr and then harvested. Data represent the mean  $\pm$  SEM of values obtained from 5 mice per group. No statistical differences were obtained between groups ( $p > 0.5$ ) as determined by ANOVA. To control for non-specific effects, the responses of cells to ConA were also measured. ConA (5  $\mu$ g/ml) responses varied between  $230,000 \pm 28,000$  and  $270,000 \pm 35,000$  (mean  $\pm$  SEM) CPMs and there were no significant differences between groups.





**Figure 32. *In vitro* proliferation of popliteal lymph node cells from immunized mice.** The remainder of the legend is as for Figure 31. Significant differences were obtained between: TBS vs rLHSP60 and IL-12 ( $P=0.01$ ), TBS versus rLHSP60 ( $P=0.01$ ), IL-12 versus rLHSP60 ( $P=0.01$ ), and IL-12 versus rLHSP60 plus IL-12 ( $P=0.01$ ) as determined by the T-KMC test. The responses of cells to ConA (5  $\mu\text{g/ml}$ ) varied between ( $180,000 \pm 22,000$ ) and ( $220,000 \pm 15,000$ ) (mean  $\pm$  SEM) CPMs and there were no significant differences between groups.

### 3. Discussion

HSP60 has been referred to as a 'common antigen' because antibodies against this protein are commonly found in a variety of human infections that involve bacteria, protozoa and metazoa. HSP60 is also a commonly recognized T cell antigen in mycobacterial infections. Mycobacterial HSP60-specific T cell lines and clones have been isolated from both tuberculosis and leprosy patients and from healthy donors (44,106). Evidence also indicates that the HSP60 can confer protection against intracellular infections in animal models. Thus, experiments in which immunization was carried out using HSP60 as antigen (164,167,168,171) or in which HSP60-specific T cells were adoptively transferred into irradiated, naive animals (170) demonstrated that HSP60 can engender protective immune responses against subsequent challenge infections.

In the present study, recombinant LHSP60 was used to determine whether antibodies to this protein are present in sera of patients with leishmaniasis. Patients with American cutaneous leishmaniasis from an endemic zone in Colombia were found to contain high levels of serum antibodies against LHSP60. Since it is known that anti-leishmania antibodies do not protect against infection with these organisms, the significance of the presence of antibodies to LHSP60 is unclear. In contrast to LHSP60, levels of antibodies to mycobacterial HSP65 were not elevated in sera from these patients. Thus, there appears to be an active and selective, humoral immune response to LHSP60 in infected individuals. Selective recognition of LHSP60 suggests that these antibodies are likely directed against non-conserved regions of the molecule. Mice immunized with mycobacterial HSP65 develop antibodies that cross-react with *E. coli* GroEL, but not with human HSP60 (303), demonstrating again that humoral responses to non-universally conserved sequences will always occur. It is possible that the presence of antibodies to LHSP60 may have value in serodiagnosis. For this to be the case, however, rLHSP60 or a fragment thereof would have to be shown to be recognized in at least a genus-specific manner.

Results from this series of experiments also suggest that immunization of susceptible mice with rLHSP60 in the absence of adjuvant mitigates disease early after challenge with *L. major*.

Thus, Balb/c mice immunized with rLHSP60 and then infected with leishmania promastigotes have significantly smaller lesion sizes as compared with control animals (recipients of TBS alone) (Figures 27 and 28). This effect was found to persist through five weeks of infection, at which point (presumably) the inflammatory response in rLHSP60 immunized mice began to increase. By week seven lesion sizes were the same in this group and in animals treated with TBS alone. That this early protective effect was the result of immunization with rLHSP60 is supported by the finding that while IL-12 immunized mice had the largest lesion sizes during the first five weeks, this effect was significantly reduced when mice were immunized with rLHSP60 plus IL-12 (Figures 27 and 28). This suggests that the early protective effect of rLHSP60 may have counteracted the early exacerbating action of the IL-12.

The basis for the early mitigating effect of rLHSP60 is unclear. It may be that activation of macrophages by rLHSP60 results in enhanced killing of ingested organisms. This possibility is supported by recent research suggesting that HSP60 may enhance the innate immune response through a mechanism that involves macrophage activation. In fact, peritoneal macrophages explanted from HSP65-treated C57BL/6 or CBA/J mice, release more  $H_2O_2$ , inhibit proliferation of *T gondii*, and kill *L. monocytogenes* more effectively than do peritoneal macrophages from untreated mice (304). Furthermore, inhibition of proliferation of *T gondii* correlates with enhanced HSP65-induced production of  $TNF-\alpha$ , IL-6 and reactive nitrogen intermediates by peritoneal macrophages from C57BL/6 naive mice (166). In light of these data, results of the present study suggest that macrophages of rLHSP60 immunized mice may have become activated. These cells would have the potential to enhance killing of ingested organisms thereby contributing to early control of disease.

Contrary to the finding that immunization with rLHSP60 results in early protection or disease mitigation, other results indicate that long-term protection is not conferred by this protein using this specific immunization protocol. Thus, an abrupt increase in lesion size was observed after five weeks of infection and by seven weeks, parasite numbers in lesions of these animals were only slightly lower than those observed in controls. These results suggest that acquired protective immunity involving LHSP60-specific  $CD4^+$  T cells of Th1 phenotype did

not develop in rLHSP60 immunized mice using this immunization protocol. While lymph node cells taken from these mice did proliferate significantly in response to rLHSP60 (Figure 32,  $p=0.001$  by ANOVA), the significance of the presence of these responding T cells is not known. They may have developed de novo in response to immunization or they may represent simply an expansion of preexisting HSP60-reactive T cells which are known to be normally present under basal conditions in mice (163). Although these cells did proliferate in response to LHSP60, unless they also produce predominantly IFN- $\gamma$  and IL-2 they would not be expected to be protective.

The lack of a long-term protective effect in rLHSP60 immunized Balb/c mice against *L. major* infection, may be a consequence of the absence of an appropriate adjuvant that could focus the immune system into a Th1 type cell response while at the same time providing slow release of antigen. For example, *L. pneumophila* HSP60 administered to guinea pigs in complete Freund's adjuvant (CFA) with subsequent boosting in conjunction with incomplete Freund's adjuvant (IFA) protects 86% of animals against infection with these organisms (167). Similarly, HSP70 from *H. capsulatum* administered with CFA significantly protects C57BL/6 mice against subsequent challenge infection (172). In the present study, immunization with rLHSP60 without adjuvant may have led to relatively rapid elimination of the protein from the system. As such, antigen may have not been available for processing and presentation to HSP60-specific T cells for a sufficient period of time to engender long-lasting immunity.

Surprisingly, immunization with rLHSP60 in combination with IL-12 as adjuvant did not confer protection as determined by lesion size at seven weeks (Figure 29), although parasite numbers in lesions were reduced at this time (Figure 30). However, the latter effect was also seen with IL-12 alone. It has been shown that vaccination of Balb/c mice with IL-12 as adjuvant together with either a soluble leishmania antigen (SLA) or a 36-kDa leishmania antigen (LACK) promotes CD4<sup>+</sup> Th1 cell development and resistance to subsequent infection with *L. major*. These effects were assessed by determination of both lesion size and numbers of parasites in lesions (213,241). Quantitation of organisms in lesions showed that mice immunized with either SLA alone or IL-12 alone contained  $\sim 10^7$  organisms/lesion in

comparison to control mice which had  $10^8$  parasites. In contrast, Balb/c mice immunized with SLA plus IL-12 has  $10^3$  total organisms per lesion (213). These results indicate that in this model system, IL-12 alone did not exert substantial control of parasite loads.

These findings are at variance with those of the present study. Thus, at seven weeks of infection, Balb/c mice that had been immunized with either rLHSP60 or IL-12 contained respectively  $10^9$  and  $10^5$  parasites per lesion compared to control mice with  $10^{12}$  organisms. Mice immunized with rLHSP60 plus IL-12 also had  $10^5$  parasites per lesion. Thus, immunization with IL-12 had a significant effect ( $p < 0.05$ ) in reducing parasite loads at 7 weeks of infection, but LHSP60 did not. Under these conditions, the protective potential of rLHSP60 in the presence of IL-12 may have been obscured by the potent antiparasitic activity of IL-12. Further studies of the vaccine potential of rLHSP60 if any, will require the development of appropriate conditions for effective immunization and may involve and alternative adjuvants.

## X. GENERAL DISCUSSION

### A. Expression of HSPs in stressed cells

Survival of intracellular pathogens within macrophages requires that they are able to withstand hostile conditions including, but not limited to acidic pH, toxic oxidative products, lysosomal proteases and elevated temperatures. On the other hand, the process of microbial invasion and intracellular replication very likely provokes changes within the macrophage which influence the outcome of infection. Knowledge about the expression of both microbial and host factors within macrophages and how they are regulated will undoubtedly contribute to an understanding of intracellular survival. In the case of *Salmonella* for example, infection of murine macrophages is accompanied by increased synthesis of specific salmonella proteins. This occurs within 30 min of infection and continues for 20 hr during the active phase of bacterial growth. In contrast, the synthesis of certain other salmonella proteins within macrophages is substantially reduced or completely terminated (96).

Normal murine BMM infected with *M. bovis* are recognized by mAb IA10 raised against *M. bovis* HSP65. This antibody also recognizes a 68-70 kDa protein in macrophages treated with IFN- $\gamma$ . Thus, molecules similar to mycobacterial HSP-65 are expressed in infected BMM (142). Whether they are of host or microbial origin is as yet unclear. Monoclonal antibody IA10 also recognizes a 65-kDa molecule in peritoneal macrophages obtained from mice infected with *T. gondii*. Here again, the precise origin of HSP65-like molecule is not known (168).

Metabolic labeling with [ $^{35}$ S]-methionine identified at least 16 *M. tuberculosis* proteins induced in infected THP-1 cells. Many of these proteins expressed intracellularly were also found to be expressed by treating bacteria extracellularly with either heat shock at 42 $^{\circ}$  C, low pH or H $_2$ O $_2$ . However, none of these stress treatments reproduced completely the pattern of protein expression observed intracellularly. Thus, extracellular treatment of bacteria with either heat shock, H $_2$ O $_2$  or low pH induced respectively 20, 4, and 15 proteins. Eight proteins induced within the cell were not induced under any of these extracellular conditions.

Thus, adaptation of pathogens including leishmania to the intracellular environment requires a complex set of factors that include expression of stress proteins which may favor their survival (97). For example, promastigotes of *L. major* that grow from logarithmic to stationary phase in culture have increased infectivity for both Balb/c mice in vivo and for murine macrophages in vitro (179,182). Stationary phase promastigotes of *L. chagasi* are also more infectious for Balb/c mice and are also more resistant to the toxic action of H<sub>2</sub>O<sub>2</sub> than are exponentially growing organisms (130, 364). Relative resistance of stationary phase promastigotes to H<sub>2</sub>O<sub>2</sub> may be related to reduced generation of hydroxyl radicals ( $\cdot$ OH) from H<sub>2</sub>O<sub>2</sub> in the presence of stationary phase organisms. Thus, it has been shown that treatment of stationary phase promastigotes with H<sub>2</sub>O<sub>2</sub> results in significantly less  $\cdot$ OH production than does similar treatment of logarithmic phase organisms (184). This is likely important since  $\cdot$ OH is a highly toxic compound derived from H<sub>2</sub>O<sub>2</sub> and has been implicated in microbe killing by macrophages (184). An alternative mechanism to explain relative resistance of stationary phase involve induction of the stress response and not involve reduced production of  $\cdot$ OH. For example, exposure of promastigotes to heat shock at 37<sup>0</sup> C results in a stress response with induction of HSP70 and development of resistance to H<sub>2</sub>O<sub>2</sub>. In this system, however, reduction of  $\cdot$ OH production by H<sub>2</sub>O<sub>2</sub>-treated parasites was not observed (184). Furthermore, this resistance may be cross-protective since exposure of promastigotes to either sublethal concentrations of H<sub>2</sub>O<sub>2</sub>, the oxidative agent menadione or heat shock at 37<sup>0</sup> C results in increased resistance to H<sub>2</sub>O<sub>2</sub>. Thus, a cross-protective stress response occurs when leishmania parasites are subjected to either heat shock or oxidative stress (247).

Research described in this thesis examined the expression of HSPs in macrophages infected with *L. donovani*. Immunoblotting with a rabbit anti-HSP60 antibody raised against moth HSP60, revealed that two proteins (65- and 67-kDa) appear de novo in infected BMM, and these heat-inducible proteins were shown to be leishmania in origin. They appear rapidly (within 3 hr of infection) and are inducible, with maximal expression observed at 24 hr. The conclusion that these two proteins are leishmania HSPs and not murine HSP60 is supported by two findings: (i) two proteins with identical electrophoretic mobilities and the same relative

abundance are found in stationary-phase leishmania promastigotes, and (ii) these proteins are not detectable in noninfected macrophages either in the basal state or after exposure to several conditions known to bring about stress protein expression.

Infection of BMM is also accompanied by the expression of a minimally induced leishmania protein of the HSP70 family. This protein has an apparent subunit size of 70-kDa and is thus essentially identical in size to murine HSP70. The latter protein was not induced to a significant extent when uninfected BMM were subjected to either NaAsO<sub>2</sub> or to phagocytosis of fixed *S. aureus*. As was the case for the 65- and 67-kDa heat-regulated proteins, leishmania HSP70 is also expressed constitutively in stationary-phase promastigotes. The findings obtained also indicate that, in contrast to the 65- and 67-kDa heat-regulated leishmania proteins, host proteins belonging to the 60-, 70- and 90-kDa HSP families do not appear to alter their expression in response to leishmania infection. This is of significant interest, as it is well known, for example, that viral infections frequently bring about increased expression of host HSPs, and it has been postulated that other intracellular infections with bacteria as well as with more complex organisms may do the same (44).

High level expression of leishmania HSP60 and HSP70 in infected macrophages suggests that these HSPs may be important during leishmania infection. Abundant levels of HSP70 and HSP83 (2.1 and 2.8% of total protein, respectively) are found constitutively in promastigotes of *L. major* and are further induced during heat shock at 37° C (250). *Leishmania* are confronted by and must withstand harsh environmental conditions as an integral part of their life cycle. It is known that extreme conditions in both pH and temperature trigger changes in gene expression in leishmania promastigotes leading to transformation into amastigote forms (188). HSP70 is known to be a molecular chaperone that binds unfolded polypeptides helping to maintain them in extended conformation until proper conditions for refolding occurs. Members of the HSP60 family also bind unfolded polypeptides and assist them in the reacquisition of native structures (1-3). Thus, leishmania HSP70 and HSP60 may be involved in the renaturation of heat-damaged proteins that unfold when the organism is confronted by an increase in temperature in the mammalian host. Heat shock proteins may also enable rapid



adaptation of leishmania to different temperatures while cycling between invertebrate vectors ( $\sim 22$ -  $27^{\circ}$  C) and mammalian hosts ( $37^{\circ}$  C). Such a role would be consistent with the abundant expression of HSPs in both stages of the organism's life cycle (188).

In addition, leishmania HSPs may assist in coping with oxidative stress imposed by the macrophage. For example, HSPs may stabilize macromolecular complexes within the organism which may need to be reorganized after exposure to toxic ROIs and other degradative products within the macrophage. Incubation of *L. donovani* promastigotes with sublethal concentrations of  $H_2O_2$  or other oxidant generating compounds such as the superoxide-generating drug, menadione, results in enhanced expression of HSP70. At the same time, exposure of parasites to either sublethal concentrations of  $H_2O_2$ , menadione or heat shock causes these organisms to become more resistant to  $H_2O_2$ . This is indicative of a cross-protective stress response and may be dependent upon the regulated expression of HSPs (247). Leishmania HSPs may also contribute to virulence. For example, treatment of *L. chagasi* with sublethal concentrations of menadione causes promastigotes to become more virulent for Balb/c mice (247) and culture forms of *L.b.panamensis* exposed to  $34^{\circ}$  C for 9-48 hr are more infective for Syrian hamsters than are organisms cultured at  $26^{\circ}$  C (246).

Phagocytosis of microorganisms by macrophages represents a process that causes "stress" both for the pathogen and for the host cell. The latter is consistent with the finding that HO-1 is induced in BMM infected with leishmania (Figures 7-9). HO-1 is expressed 3 hr after infection and shows enhanced expression thereafter with a maximum at 24 hr. As expected, this protein was also found to be induced in response to  $NaAsO_2$  and during phagocytosis of *S. aureus* by BMM. It has been shown previously that ingestion of sheep erythrocytes by human monocytes leads to induction of heme oxygenase (89). Addition of exogenous  $H_2O_2$  or *S. aureus* to human mononuclear phagocytes also induces synthesis of heme oxygenase (80,89) as well as HSP70 (80,289). Ingestion of *S. aureus* also brings about induction of superoxide dismutase (80). Thus, induction of HSP70 as well as the scavenger molecules HO-1 and superoxide dismutase suggests that these molecules may be involved in protection against ROI generated during phagocytosis and oxidative injury to host cells.

Previous studies concerned with HO-1 expression have not addressed induction of this enzyme in macrophages infected with *Leishmania*. HO-1 is a stress protein induced by hemin as well as by a variety of other oxidative stressors including exposure to heavy metals or ionizing radiation (85). Although the exact mechanism of how HO-1 protects against oxidative stress is not known, it is believed that it may do so at least in part through the reaction products it catalyzes. HO-1 catalyzes the conversion of heme to biliverdin and its reduced product bilirubin, both of which are effective scavengers of ROI (85). The finding that host HO-1 is induced in cells infected with *Leishmania* is consistent with an anti-oxidative stress response taking place during leishmania infection of macrophages.

### **B. Immune recognition of HSP60**

The rapid and high level expression of inducible, leishmania HSP60-related proteins in infected macrophages suggested that these proteins may be targets of the immune response during the leishmaniasis. To facilitate further studies of LHSP60 including leishmania disease pathogenesis and its potential role in vaccine development, the gene encoding the LHSP60 was cloned, sequenced and expressed in *E. coli*.

*L. major* HSP60 was found to be a 1770 bp single copy gene encoding a putative leishmania HSP60 of 589 amino acids with a predicted size of  $M_r$  64,790. Comparison of the predicted LHSP60 amino acid sequence with other eukaryotic HSP60 sequences indicated that similar to *T. cruzi* HSP60, LHSP60 contains a mitochondrial targeting sequence located at the N-terminus. In contrast to most HSP60s, LHSP60 does not contain the repeat GGMGGM motif at the C-terminus which is characteristic of this family of proteins. LHSP60 was also found to possess significant degrees of sequence identity with other eukaryotic homologs. Thus, it has 55%, 47%, 49% identity respectively with *T. cruzi*, human and *M. leprae* HSP60s. However, similar to HSP60 from *P. falciparum*, the C-terminus of LHSP60 diverges substantially from other HSP60s including the human homolog. Furthermore, throughout the coding sequence, there are regions of significant sequence dissimilarity between

LHSP60 and human HSP60. Since HSP60 is highly immunogenic, these unique regions could represent potential T cell epitopes involved in an active leishmania infection (see below).

From an immunological perspective, the high degree of conservation of HSP sequences across species of invading pathogens has been regarded as potentially beneficial to the host. Since many HSPs are produced at elevated levels in response to various stressful conditions, they are markers of "stressed" cells which constantly arise during host-pathogen interactions. Immune recognition of these proteins may, therefore, contribute to immune surveillance (128). Macrophages subjected to "stress" stimuli including IFN- $\gamma$  and infection with murine cytomegalovirus for example were found to be recognized and lysed by mycobacterial HSP65 specific CD8<sup>+</sup>  $\alpha/\beta$  T lymphocytes (CTLs). Through recognition of conserved epitopes in HSP60, T cells such as these may contribute to the first line of defense against a wide variety of infections by destroying "stressed" cells harboring growing pathogens (142).

On the other hand, recognition by T cells of regions of high sequence similarity between host HSP60 and microbial HSP60s may induce autoimmune reactivity (128). Recognition of self-epitopes may be detrimental for the host when it becomes chronic or exacerbated due to long term stimulation in cases of persistent inflammation or infection (128). For example, human and experimental animal studies suggest that the mycobacterial HSP65 may be involved in rheumatoid arthritis (106). A variety of other microbial species have also been implicated in autoimmune disease manifestations (156). Cross reactive epitopes shared between microbial and host HSPs have been identified that are recognized by  $\alpha/\beta$ -T cells (128). T cells with specificity for mycobacterial HSP65 have also been identified in both patients with tuberculosis and leprosy and in healthy individuals (106,156). No data are currently available related to the presence of leishmania HSP60-specific T cells that may contribute either to immune surveillance or autoimmunity. Nevertheless, the high degree of significant sequence similarity between LHSP60 and its homologs from other organisms including humans suggests that they likely exist.

Results reported in this thesis show that sera from patients with American Cutaneous Leishmaniasis recognize recombinant LHSP60. This same protein was not recognized to a

significant degree by sera from healthy donors. Several observations suggest that recognition of rLHSP60 by sera from patients with leishmaniasis may be specific: (i) in contrast to LHSP60, antibodies to mycobacterial HSP65 were not elevated in patient sera, (ii) since LHSP60 shows 64% similarity (identical plus conservative amino acid replacements) with its mycobacterial counterpart (Table 3), this suggests that sera from patients recognize non conserved regions of rLHSP60, (iii) remarkable sequence dissimilarity between LHSP60 and most other HSP60s is concentrated in the C-terminus (Figure 21) raising the clear potential for species-specific epitopes. In addition, it was previously demonstrated that mice immunized with mycobacterial HSP65 develop antibodies that cross-react with *E coli* GroEL (HSP60), but not with human HSP60 (303) demonstrating that humoral responses to non-universally conserved sequences are the norm.

The significance of anti-LHSP60 antibodies in patients with leishmaniasis remains to be determined. Antibodies do not appear to play a role in the resolution of leishmania infection. In fact, antibodies are increased in chronic disease, while low antibody titers are associated with healing infections (195). It has been suggested that in some cases infected macrophages may be unable to receive activating signals from T cells because of the presence of circulating leishmania antigen-antibody complexes (305). Immune complexes have been shown to impair macrophage functional properties such as IFN- $\gamma$  induced expression of MHC class II molecules (306). Immune complexes have also been shown to reduce the ability of murine peritoneal macrophages to kill intracellular *L. monocytogenes* (306). Thus, it is possible that since leishmania HSPs are abundant and immunogenic, they may be involved in HSP immune-complex formation during infection. Further studies may help to characterize the nature and function of the anti-LHSP60 antibodies in patients with leishmaniasis.

Since LHSP60 antibodies in sera from patients appear to have some degree of specificity, most probably related to non conserved sequences of the molecule (in particular the carboxy-terminus), these regions may have diagnostic value. Further research including epitope mapping may define LHSP60 specific B cell epitopes that differ from other HSP60 homologs in cases of infections caused by *T. cruzi*, *P. falciparum*, *M. leprae*, *M. tuberculosis* and

*Paracoccidioides braziliensis*. Diseases caused by these pathogens are commonly found in areas where leishmaniasis is endemic and the presence of disease specific epitopes would have significant value (284).

During the present studies, rLHSP60 was used to immunize susceptible Balb/c mice. Groups of mice were immunized on three occasions before infection with either rLHSP60, rLHSP60 plus IL-12, IL-12 alone or TBS. IL-12 was used as a putative adjuvant (213,241). Mice were subsequently challenged in the footpad with infective doses of *L. major* promastigotes and the course of the infection was monitored by weekly measurements of footpad swelling. Seven weeks after infection, mice were sacrificed for determination of parasite numbers in infected tissue and measurement of T cell responses to rLHSP60. Results demonstrated that (i) early after infection (up to five weeks), mice immunized with rLHSP60 alone develop smaller lesions compared to control animals (given TBS alone), (ii) rLHSP60 does not confer long-term protection to mice since seven weeks after infection these animals develop lesions and parasite numbers not significantly different from mice receiving TBS alone, (iii) IL-12 alone exerts potent anti-leishmania activity, (iv) IL-12 does not exhibit adjuvant activity when administered in combination with rLHSP60.

The early protective or disease mitigating effect of rLHSP60 is of interest and may be related to macrophage activation. It has been shown that peritoneal macrophages from C57BL/6 or CBA/J mice treated with mycobacterial HSP65 release more  $H_2O_2$ , inhibit proliferation of *T. gondii* and kill *L. monocytogenes* more effectively than peritoneal macrophages from untreated mice (304). Peritoneal macrophages from C57BL/6 mice treated in vitro with HSP65 from mycobacteria show enhanced inhibition of *T. gondii* proliferation and enhanced release of  $TNF-\alpha$ , IL-6 and reactive nitrogen intermediates (166). Thus, it is possible that immunization with rLHSP60 leads to macrophage activation and enhanced killing of leishmania in vivo early after infection.

The lack of a long-lasting protective effect in rLHSP60 immunized Balb/c mice may be a consequence of the absence of an appropriate adjuvant. The latter optimally would focus the immune system into a Th1 type response while at the same time providing sustained release of

antigen. In these experiments, immunization with IL-12 alone had a significant effect in reducing parasite load and lesion size at seven weeks of infection. However, immunization with IL-12 plus rLHSP60 did not result in augmented protection against infection indicating that IL-12 did not provide adjuvant activity. Previous studies showed that vaccination of Balb/c mice with the leishmania antigens SLA or LACK in combination with IL-12 promotes resistance against subsequent infection with *L. major*. This protection appears to be specifically elicited by these antigen preparations and amplified by the adjuvant effect of the IL-12. In these experiments, neither antigen nor IL-12 given alone were able to confer protection to mice (213,241). In contrast, the present experiments show that IL-12 confers an antileishmania rather than an adjuvant effect. The basis for these divergent findings is not known. IL-12 is a pleiotropic cytokine. In addition to potentiating the development of a protective cell-mediated immune response, it has also been found to have therapeutic activities. Administration of IL-12 alone to Balb/c mice infected with *L. major* beginning the same day of infection resulted in cure of ~89% animals (207). High cure rates were also observed when Balb/c mice were given IL-12 two weeks after infection with *L. donovani* (307). Thus, the characteristics that separate prophylactic versus therapeutic effects of IL-12 need to be defined further.

It has been shown previously that immunization with *L. pneumophila* HSP60 in complete Freund's adjuvant (CFA) significantly protects guinea pigs against infection with these organisms (167). Similarly, HSP70 from *H. capsulatum* administered in the presence of CFA protects mice against subsequent challenge infection (172). These results indicate that in the presence of an appropriate adjuvant, HSP60 does provide protection. Thus, further studies of the vaccine potential of rLHSP60 will require the development of other protocols for immunization, the use of alternative adjuvants or both.

The carboxy-terminal 50 amino acids of LHSP60 was found to diverge substantially from other HSP60s including the human homolog (Figure 21). T cell epitopes within this region are clearly unique and their potential for inducing protective immunity is unknown. Thus, in

future studies there may be value in using synthetic peptides derived from this region for immunization in combination with appropriate adjuvants.

rLHSP60 or peptides derived from this molecule may also be used as carriers of antigenic peptides in vaccine constructs. This possibility is based on recent research indicating that due to particular peptide-binding capabilities, some HSPs may have intrinsic adjuvant properties (120,123,124). For example, immunization of mice with the polypeptide (NANP)<sub>40</sub> (a repetitive sequence of the *P. falciparum* circumsporozoite protein) conjugated to mycobacterial HSP60 or HSP70 results in strong anti-peptide IgG antibody responses (120). Mice immunized with HSP70 derived from tumor cells are protected against challenge with lethal doses of the same tumor cells. Protection appears to be conferred not by the HSP itself, but by a bound tumor-derived peptide while an adjuvant effect appears to be provided by the chaperone (124). Immunization of Balb/c mice with HSP60 peptides containing immunogenic T cell epitopes conjugated to a poorly immunogenic peptide of *S. typhi* (Viag) induces high levels of anti-Viag antibodies. The HSP60-peptide carrier effect, in this case, is T cell dependent and is not associated with autoimmune responses (123). Thus, immunization with rLHSP60 (or derived peptides) in combination with leishmania antigens known to have vaccine potential, such as gp63, or immunogenic gp63 peptides may enhance protection against subsequent infection with *Leishmania*.

Finally, it is conceivable that the leishmania *HSP60* gene may be used to develop a live leishmania vaccine. Vaccines consisting of live attenuated organisms generally confer better protection than do killed ones and may be superior to subunit vaccines provided that safe preparations can be made (240). Vaccination with *L. major* (dhfr-ts<sup>-</sup>) produced by targeted deletion of the genes encoding dihydrofolate reductase-thymidine kinase (DHFR-TS) does not by itself induce disease and significantly protects Balb/c mice against infection with *L. major* (240). Vaccination using thermosensitive leishmania mutants also protects against challenge infections. However this approach requires genetic stability of thermosensitivity which has not yet been demonstrated (247). Thus, targeted disruption of the leishmania *HSP60* gene may

result in an attenuated strain which does not survive high temperatures (37<sup>0</sup> C) and which may engender protective immunity.

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