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Heat Shock Proteins in *Mycobacterium tuberculosis*: Involvement in Survival and Virulence of the Pathogen

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1. Introduction

Tuberculosis (TB) is an infectious disease of global concern. Worldwide TB kills two million people each year. About 90% of those infected with *Mycobacterium tuberculosis* have asymptomatic, latent TB infection (sometimes called LTBI) (Smith, 2003; Wayne & Sohaskey, 2001). Years after initial infection, the bacilli may resume growth, the outcome of which is active TB. If left untreated, the death rate for active TB cases is more than 50%. Approximately 95% of new cases and 98% of deaths occur in developing nations, where human immunodeficiency virus (HIV) infections are common, this is generally because of the unavailability of proper treatment. The causative agent, *M. tuberculosis* has a cell wall which has a very low permeability for most antibiotics and chemotherapeutic agents. Another critical problem is the development of multi-drug resistant TB (MDR-TB) or extremely drug resistant TB (XDR-TB) (Chiang et al., 2010; Eismont, 2009; WHO report, 2010). Every year in the world, around 440,000 new MDR-tuberculosis cases are found due to bacilli that are resistant to the two main antitubercular drugs, isoniazid and rifampicin. The XDR-TB is a recently developed form. The mortality rate in the case of XDR-TB can go from 50 to 100%. *M. tuberculosis* mutants, resistant to any single drug are naturally present in any large bacterial population, irrespective of exposure to drugs. Despite the availability of effective chemotherapy and the moderately protective vaccine, new anti-TB agents are urgently needed to decrease the global incidence of TB (Cox et al., 2006; Ducati et al., 2006).

2. Mycobacterial infection and survival of pathogen inside the host

On infection, *M. tuberculosis* resides mainly in the host macrophage, inside an endocytic vacuole called the phagosome. The pathogenic mycobacteria inhibit phagosome-lysosome fusion (Hestvik et al., 2005; Pieters & Gatfield, 2002). Lack of maturation of phagosomes containing pathogenic *M. tuberculosis* within macrophages has been widely recognized as a crucial factor for the persistence of mycobacterial pathogen. Mycobacteria have been shown to remain within phagosomes for a long time after infection by EM analysis (Jordao et al., 2008). It is unclear whether blocking of phagosome–lysosome fusion is essential for *M.*

tuberculosis survival (Armstrong & Hart, 1975). After phagocytosis and replication of pathogenic bacteria within macrophages, the infected cells migrate into tissues where additional immune cells are recruited to form a granuloma which consists of T cells and *M. tuberculosis*-infected macrophages (Grosset, 2003). The granuloma subsequently develops central areas of necrosis called caseum. This mass of cells of immune system and the bacteria are all dead cells. The surviving bacilli exist in a latent state and can become reactivated to develop active disease (Grosset, 2003). The latent infection in the asymptomatic individuals serves as a large reservoir of the bacterium. The biology of the latent state of the bacterium is not completely understood, however it is accepted that the latent state bacilli are metabolically less active (Wayne & Sohaskey, 2001).

Inside the macrophages, *M. tuberculosis* encounters many stress conditions like nitric oxide generated by inducible nitric-oxide synthase, nutrient starvation or carbon limitated condition, and reactive oxygen species (ROS) by the phagosomal NADPH oxidase (Farhana, 2010; Ehrt, 2009; Butler, 2010; Beste, 2007; Axelrod, 2008). A large number of studies have been undertaken to understand the survival of *M. tuberculosis* under stress such as heat, reduced oxygen or hypoxia, nutrient starvation, reactive nitrogen intermediates (RNI), antimicrobial molecules and downshift in pH (Chan et al., 1992; Farhana et al., 2010; Firmani & Riley, 2002; Lowrie, 1983; Wayne & Sohaskey, 2001). It has been suggested that the bacteria enter the non-growth or stationary phase during such stress conditions (Wayne & Sohaskey, 2001). *M. tuberculosis* also survives the lethal effects of RNI and antimicrobial molecules produced by activated macrophages and other cell types (Chan et al., 1992). The intracellular pathogen has the ability to survive inside the host macrophage in spite of the microbicidal effector functions of the macrophages. The bacterium responds to the stress conditions by genome wide changes in gene expression including the induction of a transient expression of a well conserved set of genes encoding heat shock or heat stress proteins.

3. Heat shock proteins

Heat shock proteins (Hsps) are well conserved and universal in all organisms. The expression of Hsps is highly increased under stress conditions such as hypoxia, nutrient starvation and oxygen radical (Richter et al., 2010; Tyedmers et al., 2010). Heat shock and other types of stresses lead to protein aggregation and unfolding of proteins. However, the most deleterious effect is the collapse of intermediary filament, tubulin and actin networks which leads to complete loss of localization and breakdown of intracellular transport and fragmentation of ER (Toivola et al., 2010). Hsps have cytoprotective roles, and under the stress conditions they maintain the cellular organization and homeostasis. Hsps are expressed at significant level in all eukaryotic and prokaryotic cells under normal conditions at physiological temperature. There is a high level of conservation of Hsps indicating a fundamental role played by these proteins in cellular processes (Moseley, 1997; Richter et al., 2010). Hsps were initially discovered in *Drosophila melanogaster* larvae as chromosomal puffs when it was exposed to heat shock (Tissieres et al., 1974). Subsequently, several Hsps were discovered in the following years. Many heat shock proteins work as molecular chaperones that are essential for maintaining cellular functions by preventing misfolding and aggregation of nascent polypeptides and by facilitating protein folding of conformationally altered proteins (Lanneau et al., 2010; Tyedmers et al., 2010).

The predominant class of Hsps is of molecular chaperones (Ellis & Hemmingsen, 1989). The molecular chaperones are further grouped into five major families based upon their molecular masses. These families are Hsp100, Hsp90, Hsp70, Hsp60 and small heat shock protein (sHsps) (Richter et al., 2010). The classification is based on their related functions and sizes, using the conventional nomenclature adopted after the Cold Spring Harbor Meeting of 1996 (Hightower & Hendershot, 1997). The molecular chaperones not only facilitate the proper folding of proteins but many times direct improperly folded proteins for destruction. In recent years, multiple chaperone-assisted degradation pathways have emerged, in which chaperones associate with a protease present inside the cell to degrade a misfolded protein (Gottesman, 2003, Kettern et al., 2010). Several other small heat inducible molecular chaperones, like Hsp33 are also known (Jakob et al., 1999).

The Hsp100 family consists of a group of ATPases associated with cellular activities (AAA+) family of ATP-dependent chaperones that transfers aggregated protein into a proteolytic chamber of an associated protease. These energy-dependent proteases, also known as caseinolytic proteases, Clp or Ti, are involved in a number of cellular activities, such as the degradation of proteins misfolded as a result of various types of stresses, the regulation of short-lived proteins and the housekeeping removal of dysfunctional proteins, which include denatured and aggregated polypeptides (Gottesman et al., 1997a, Gottesman et al., 1997b). The members of Hsp100 family include ClpA, ClpB, ClpC, ClpE, ClpX, ClpY and others (Kirstein et al., 2009). Hsp100 proteins have either one or two copies of a conserved ATPase, AAA+ core domain. Hsp100 family is further divided into two subclasses. The class I family members that include ClpA-E and L, contain two ATPase domains. The class II family members contain one ATPase domain, and include ClpX and ClpY (Lindquist & Craig, 1988; Schirmer et al., 1996). The Clp proteins form hexameric structure with one nucleotide binding site in each monomer of Class II and two nucleotide binding sites in Class I (Schirmer et al., 1996). These ATP-dependent chaperones associate with a protease, ClpP or ClpQ forming an oligomeric enzyme which assembles into ring-like or barrel like structure, containing a cavity within the centre of the macromolecular structure (Gottesman, 2003). The central cavity is also known as the proteolytic chamber, where unfolded protein substrates are translocated and subsequently degraded by the proteolytic site (Gottesman, 2003). Degradation of structured protein substrates requires the presence of ATP (Baker & Sauer, 2006). Unlike the other class I Clp proteins, ClpB does not associate with any protease to direct substrates for degradation (Lee et al., 2004).

Hsp90 is present mostly in cytosol of bacteria and eukaryotes, and is upregulated under stress (Welch & Feramisco., 1982). This chaperone is different in a way that it is not very promiscuous in substrate binding as it does not bind unfolded proteins rather it binds to native like proteins (Jakob et al., 1995). Under stress conditions two of the Hsp90 family proteins, namely yeast Sti1 and the propyl isomerise, Cpr6 are upregulated (Pearl & Prodromou., 2006).

Hsp70 family consists of highly conserved chaperones. All Hsp70 proteins bind ATP and under physiological conditions prevent the aggregation of proteins, and also refold aggregated proteins (Kiang & Tsokos, 1998). The activity of Hsp70 is regulated by co-factors. Much of the functional diversity of Hsp70s is driven by a diverse class of cofactors named J proteins or Hsp40 (Kampinga & Craig, 2010). The major members of the Hsp70 family include HSC 70 (heat shock cognate 70), mitochondrial GRP 75 and GRP 78 (Shi & Thomas,

1992). Hsp70 proteins in the endoplasmic reticulum are involved in two distinct chaperone functions in the normal cell. In the first, the Hsp70 family chaperone transfers the newly synthesized, unfolded protein to Hsp60 family of chaperonins, leading to eventual folding of the proteins. In the second case, Hsp70 chaperones carry proteins to different cellular compartments for the proper folding of the proteins (Kiang & Tsokos, 1998, Shi & Thomas, 1992).

Chaperonins are ring shaped proteins involved in promoting the ATP dependent folding of proteins under normal as well as under stress conditions. GroE machinery is the most prominent chaperonin in bacteria (Horwich et al., 2006). It consists of 14 GroEL subunits arranged in a cylinder of two heptameric rings, which is further attached to a heptameric ring of GroES (Horwich et al., 2006). GroE can bind to several different types of non-native proteins. The non-native protein is encapsulated in the GroE cylinder. GroEL internalizes the protein for the length of ATP hydrolysis cycle, during which the protein can refold to its native state (Viitanen et al., 1992). The closely related proteins in the mitochondria are called as Hsp60 and Hsp10.

Small Hsps (sHsps) are the most poorly conserved group among Hsps. Their most common trait is an α-crystallin domain. The most prominent member is the eye lens protein α-crystallin or Acr (Horwitz, 2003). sHsps are ATP-independent chaperones that form a large oligomeric structure often composed of 24 subunits. sHsps interact with partially folded targeted proteins to prevent their aggregation under stress conditions (Haslbeck et al., 2005). sHsp are also shown to be important in protecting the cell against the numerous injuries like heat stress, oxidative stress and apoptosis inducing factors (Arrigo, 1998).

4. Hsps and virulence in pathogenic microorganisms

Hsps play a central role in managing the damaged or aggregated proteins inside the cells. They have been linked to the virulence of several pathogenic microbes. *Candida albicans* expresses a bonafide heat shock response that is regulated by the evolutionarily conserved, essential heat shock transcription factor Hsf1. Hsf1 is thought to play a fundamental role in thermal homeostasis, adjusting the levels of essential chaperones to changes in growth temperature (Brown et al., 2010). In *Plasmodium falciparum* heat shock protein 70 is thought to play an essential role in parasite survival and virulence inside the host; Hsp70 is also being tried as a target for designing potential anti-malarial drugs (Cockburn et al., 2010). *Histoplasma capsulatum* is the causative agent of histoplasmosis in humans. A 62 kDa Hsp (Hsp60) of *H. capsulatum* is an immunodominant antigen which has been shown to play an important role in the adaptation of the fungus to temperature stress (Guimaraes et al., 2010). *Staphylococcus aureus* and *Staphylococcus epidermidis* can cause serious chronic infections in humans. An important factor involved in the pathogenesis of *S. aureus* is its ability to be internalized by phagocytes thereby evading the host immune system. Heat shock cognate protein, Hsc70 was identified as playing an important role in the internalization mechanism of *S. aureus* (Hirschhausen et al., 2010). ClpB gene from *Enterococcus faecalis* is linked to thermotolerance and virulence of the bacteria (de Oliveira et al., 2010). The Clp proteases appear to be critical for cell development in *Caulobacter crescentu*, and stress induction in *Bacillus subtilis* (Gerth et al., 2004). ClpC has been linked to the tight regulation of virulence genes in *Listeria monocytogenes*; it has been shown to be required for adhesion and invasion of the pathogen (Nair et al., 2000). ClpC has also been shown to be important for the

virulence and survival of *L. monocytogenes* in macrophages (Rouquette et al., 1998). In *Salmonella typhimurium* the Clp protease, ClpP is involved in maintaining the level of Sigma factors inside the bacterium; disruption of ClpP leads to decreased virulence in mice (Webb et al., 1999). ClpP mutation significantly attenuated the virulence of *Streptococcus pneumoniae* in murine intraperitoneal infection model (Kwon et al., 2003). Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar typhimurium results in loss of virulence in mice; these mutants were more sensitive to the intracellular environment of the macrophage (Gahan & Hill, 1999).

5. Hsps in *M. tuberculosis*

The ability of *M. tuberculosis* to survive under oxidative stress *in vivo* is an important aspect of its pathogenesis. Heat shock proteins are essential molecular chaperones for maintaining cellular functions during normal as well as stress conditions. The heat shock proteins also play a role in antigen presentation, and activation of lymphocytes and macrophages (Tsuchiya et al., 2009). The virulence of mycobacterium is dependent upon multiple genes that are expressed for the successful survival of the pathogen inside the macrophage. Expression of many heat shock proteins have been shown to increase under stress conditions in *M. tuberculosis* (Monahan et al., 2001; Sherman et al., 2001; Stewart et al., 2002; Voskuil et al., 2004). Proteome analysis of *M. tuberculosis* showed increased expression of Hsps such as 16 kDa α-crystallin (HspX), GroEL-1 and GroEL-2 inside macrophages. Hypoxia and starvation induce stationary phase in *M. tuberculosis*, under these conditions there is increased expression of hspX and acr2 (Sherman et al., 2001; Voskuil et al., 2004). Exposure of *M. tuberculosis* to heat shock induced the expression of hsp70 regulon, groEL, groES and acr protein (Stewart et al., 2002). The deletion of HspR, a repressor of Hsp70 proteins in *M. tuberculosis* has important impact on virulence. A HspR deletion mutant over-expressed Hsp70 proteins, and was fully virulent in the initial stages of infection; however the ability of the bacteria to establish a chronic infection was impaired as compared to the wild type (Stewart et al., 2001). The expression of Hsp65 and Hsp71 of *M. bovis* was increased under heat shock (Patel et al., 1991).

The synthesis of Hsps is increased after infection, some of which are immunodominant antigens in *M. tuberculosis* and *M. leprae* (Young et al., 1988). Hsp70 is an immunodominant antigen in *M. tuberculosis, M. leprae, Leishmania donovani, Plasmodium faciparum and Trypanosoma cruzi* (Kaufmann, 1994; Kiang & Tsokos, 1998). The heat shock protein, DnaK and many other proteins show increased expression during survival in carbon-starved stationary phase in *Mycobacterium smegmatis* (Blokpoel et al., 2005). In addition to a significant role in immune response, Hsps may also play a direct role in the virulence of *M. tuberculosis.* The over-expression of Hsps in *M. tuberculosis* leads to a better survival at higher temperature as compared to the wild type because of the protective effect of higher levels of Hsp (Stewart et al., 2001). Heat shock protein 22.5 (Hsp22.5) is a member of heat shock regulon which was shown to be activated under stress conditions, including survival in macrophages and during the late phase of chronic tuberculosis in murine lungs (Abomoelak et al., 2010). Deletion of Hsp22.5 resulted in the modulation of transcription of important genes like dormancy regulon, ATP synthesis, respiration, protein synthesis, and lipid metabolism (Abomoelak et al., 2010). Heat shock in *M. tuberculosis* has been shown to induce the expression of Acr2, a novel member of the α-crystallin family of molecular

chaperones (Wilkinson et al., 2005). The expression of acr2 increased within 1 h after infection of monocytes or macrophages. A deletion mutant (Δacr2) was unimpaired in log phase growth and persisted in IFN-γ-activated human macrophages (Wilkinson et al., 2005). GroES, also known as cpn.10 is found as a major constituent in the culture filtrate of *M. tuberculosis*, suggesting that it is exposed to the intraphagosomal milieu; it may be playing an important role in the survival of bacteria inside the phagosome (Sonnenberg & Belisle, 1997). Wayne and Sohaskey (2001) suggested that the decreased effectiveness of rifampin in the non-replicative state could be because of the stabilizing effect of chaperonin. Thus, a combination therapy of rifampin and a chaperonin inhibitor has the potential to shorten the therapeutic regimen. CD43, a large sialylated glycoprotein found on the surface of haematopoietic cells is involved in efficient macrophage binding and immunological responsiveness to *M. tuberculosis*. *M. tuberculosis* employs Cpn60.2 (Hsp65, GroEL), and to a lesser extent DnaK (Hsp70) as an adhesin that binds CD43 on the macrophage surface (Hickey et al., 2010). The crystal structure of the chaperonin 60 of *M. tuberculosis*, also called Hsp65 or chaperonin 60.2 has been solved (Qamra & Mande, 2004). Another *M. tuberculosis* small heat shock protein 16.3 (Hsp16.3) accumulates as the dominant protein in the latent stationary phase of tuberculosis infection and its expression is increased in response to stress (Valdez et al., 2002). It contains the core 'α-crystallin' domain found in all sHsps and protects against protein aggregation *in vitro* (Valdez et al., 2002). Protein phosphorylation is frequently used by organisms to adjust to environmental variations. Hsp16.3 and Hsp70 are immunodominant proteins synthesized during the *M. tuberculosis* infection. It was shown that these Hsps possess autophosphorylation activity (Preneta et al., 2004). *M. tuberculosis* genome has revealed the presence of heat shock proteins ClpP1, ClpP2, ClpC1, ClpX and ClpC2. The ClpC1 of *M. tuberculosis* has been shown to have an inherent ATPase activity, and to prevent protein aggregation as a chaperone in the absence of any adaptor protein (Kar et al., 2008). *M. tuberculosis* ClpC1 has also been shown to interact with ClpP2 (Singh et al., 2006). *M. tuberculosis* ClpC1 has been shown to interact with ResA, an anti sigma factor which is degraded by ClpC1P2 protease *in vitro* (Barik et al., 2010). Knockdown of ClpC1 in *M. smegmatis* and *M. tuberculosis* showed inhibition of RseA degradation indicating a regulatory role of Clp proteins in *M. tuberculosis* (Barik et al., 2010). ClpX is predicted to be essential for *in vivo* survival and pathogenicity and is conserved in *M. tuberculosis, M. leprae, M. bovis* and *M. avium paratuberculosis* (Ribeiro-Guimaraes & Pessolani, 2007). ClpX of *M. tuberculosis* was not able to substitute ClpC1 in ClpC1P2 protease complex (Barik et al., 2010). Knockdown of ClpX in *M. smegmatis* did not prevent the degradation of RseA indicating that ClpP2 does not associate with ClpX for its proteolytic activity (Barik et al., 2010). Leprosy and tuberculosis patients with active disease had shown the presence of antibodies recognizing ClpC in dot ELISA (Misra et al., 1996). The expression of ClpX was found to be upregulated in *M. tuberculosis* upon macrophage infection (Dziedzic et al., 2010). FtsZ is a protein known to assemble at the midcell division site in the form of a Z-ring. It is crucial for initiation of the cell division process in eubacteria. ClpX has been shown to interact with FtsZ in *M. tuberculosis* (Dziedzic et al., 2010). The crystal structure of *M. tuberculosis* caseinolytic protease, ClpP1 showed a disordered conformation of the residues in the catalytic triad, which makes the protein inactive (Ingvarsson et al., 2007). ClpP of *M. tuberculosis* has been studied as a target for drug designing (Tiwari et al., 2010). *M. avium*, the causative agent of paratuberculosis (Johne's disease) and an economic problem for beef,

dairy and sheep industries showed increased expression of ClpB gene during infection (Hughes et al., 2007). ClpB, of *M. bovis* BCG showed reactivity with sera of TB patients suggesting it to be an antigen target of the human immune response to mycobacteria (Bona et al., 1997). There are specific transcription factors that are involved in the regulation as well as transcription of Hsps during different conditions. ClgR, a clp gene regulator of *M. tuberculosis* activates the transcription of at least ten genes, including four that encode protease systems ClpP1/C, ClpP2/C, PtrB and HtrA-like protease Rv1043c, and three that encode chaperones Acr2, ClpB and the chaperonin Rv3269 (Estorninho et al., 2010). This transcriptional activation and regulatory function of ClgR is very important in the replication of bacteria inside the macrophages. It has been shown that ClgR deficient *M. tuberculosis* is not able to resist the pH inside macrophage post infection (Estorninho et al., 2010). The mechanism by which mycobacteria return to a replicating state after a non-replicating state, when exposed to low oxygen tension conditions is not clearly understood. ClgR is also implicated in the resumption of replicating state after hypoxia in *M. tuberculosis* (Sherrid et al., 2010).

6. Hsps as antigens in *M. tuberculosis*

Hsps may be released extracellularly upon necrotic cell death or independent of cell death. The mechanism of the release of Hsps is not clear (Tsan & Gao, 2004). The human as well bacterial Hsps stimulate immune response. The bacterial Hsps might modulate immunity by rapidly and directly increasing cytokine production in macrophages. T cells reacting to Hsp65 appear to play an important role in the control of *M. leprae* infection (de la Barrera et al., 1995). Hsp65 directly activates monocytes during mycobacterial infection. It leads to the production of TNF (tumor necrosis factor), IL-6 and IL-8. These cytokines are important in developing antigen specific T-cell mediated host immunity (Friedland et al., 1993). The murine intraepithelial lymphocytes (IEL), when exposed to soluble extract from *M. tuberculosis* showed elevated expression of IL-3, interferon-γ and IL-6 (Mendez-Samperio et al., 1995). Peripheral blood mononuclear cells (PBMC) from TB patients showed proliferative response to the Hsp65 (Mendez-Samperio et al., 1995). The *in vitro* immune responses to *M. tuberculosis* Hsp65 were checked in TB patients, and their PBMC showed high IFN-γ levels (Antas et al., 2005). When guinea pigs were vaccinated or infected with *M. bovis* (BCG) and virulent *M. tuberculosis*, cellular and humoral immune responses to mycobacterial stress proteins Hsp65 and Hsp70 were detected (Bartow & McMurray, 1997).

The C-terminal portion of heat shock protein Hsp70 was shown to be responsible for stimulating Th1-polarizing cytokines in human monocytes to produce IL-12, TNF-α, NO, and C-C chemokines (Wang et al., 2002). Hsp70 induces the expression of IL-10 and inhibits T-cell proliferation *in vitro*. Hsp70 appears to have immunosuppressive properties rather than inflammatory potential (Motta et al., 2007).

Hsp71 and Hsp65 are the major active components of the soluble extract of *M. tuberculosis.* Murine IELs were induced to divide and to secrete cytokines by Hsp71 and Hsp65 (Beagley et al., 1993). *M. tuberculosis* Hsp70, *M. leprae* Hsp65, and *M. bovis* BCG Hsp65 increased the levels of cytokines IL-1α, IL-1β, IL-6, TNFα, and GMCSF in macrophages (Retzlaff et al., 1994). *M. tuberculosis* contains multiple genes encoding Cpn 60 proteins, and these chaperonins have been involved in directly activating human monocytes and vascular

endothelial cells. Among them the Cpn 60.2 protein activates human PBMCs by a CD14-independent mechanism, whereas Cpn 60.1 is partially CD14 dependent and Cpn 60.1 is a more potent cytokine stimulator than Cpn 60.2 (Lewthwaite et al., 2001). Cpn 60.1 is said to play more important role in *M. tuberculosis* virulence than Cpn 60.2 (Lewthwaite et al., 2001).

M. tuberculosis can manipulate and inhibit the host response to ensure survival within macrophage. The anti-inflammatory cytokine IL-10 is shown to inhibit phagosome maturation in macrophages infected with *M. tuberculosis* (O'Leary et al., 2010). HspX/Acr is among the dormancy regulon whose expression is increased in hypoxia and on nitric oxide exposure essential for the survival of bacteria during persistence *in vivo*. Acr is also an immunodominant antigen during infection (Roupie et al., 2007). The T cells primed with Hsp65 of *M. tuberculosis/M.bovis* showed a response to the epitpoes shared by human Hsp65 and mycobacterial Hsp65, demonstrating that if activated these T cells can develop autoimmunity (Munk et al., 1989). The immune system, once primed for one Hsp might recognize other Hsps as well. Sarcoidosis (SA) is a multisystem granulomatous autoimmune disorder. The clinical and histopathological pictures of SA and TB are similar. *M. tuberculosis* heat shock proteins have been considered as proteins involved in the genesis of SA (Chen & Moller, 2008; Rajaiah & Moudgil, 2009). *M. tuberculosis* Hsps are also proposed to have a role in apoptosis which might be important in the pathogenesis of SA and TB granuloma formation (Dubaniewicz et al., 2006a; Dubaniewicz et al., 2006b). There is high cross reactivity between human and mycobacterial Hsp65. This could be the reason for the development of SA, however another hypothesis is that the BCG vaccination can develop autoimmunty in a pre-disposed host (Dubaniewicz, 2010). T-cells from SA patients produced a CD4+ response to multiple mycobacterial antigens including Hsps. These T cells were present at the site of active SA inside the human body (Oswald-Richter et al., 2010). The sera of the patients of rheumatoid arthritis (RA) showed increased levels of IgG and IgA antibody to the mycobacterial Hsp65 (Tsoulfa et al., 1989). The synovial membrane from rats and humans with arthritis appeared positive for mycobacterial Hsp65 showing the possible role of mycobacterial antigens in autoimmune diseases like arthritis (de Graeff-Meeder et al., 1990; Karopoulos et al., 1995). A survey of antigen-specific antibody isotypes from rheumatoid patients showed that anti-mycobacterial Hsp65 antibodies clearly do not appear to be disease specific markers for RA; however this does not exclude the possibility of mycobacterial Hsp65 in the pathogenesis of RA (Lai et al., 1995; Minota, 1997). The mycobacterial 71kDa Hsp antigen in lower concentration inhibits arthritis and at higher concentrations completely protects rats from arthritis (Kingston et al., 1996). Mycobacterial Hsp65 has also shown cross-reactive epitopes of epidermal cytokeratins which is a protein from epidermal keratinocytes of the normal human skin (Rambukkana et al., 1992). *Mycobacterium paratuberculosis* Hsp65 has been implicated as a possible cause of Crohn's disease, an inflammatory bowel disease (el-Zaatari et al., 1995).

As seen in several studies, Hsp65 is involved in the development of autoimmune response because of its highly conserved sequence. Another important finding about Hsp65 is that the effector cells activated with Hsp65 strongly inhibited colony formation from live BCG-infected autologous macrophages (Ab et al., 1990). In the case of *M. lepare* infection, the T-cells from leprosy patients are exposed to a large variety of different antigens including *M. lepare* Hsp70, *M. tuberculosis* Hsp70 and Hsp65 (Janson et al., 1991). When patients with

multiple sclerosis and tuberculosis were assessed, it was seen that Hsp70 is an antigen in TB as well as an autoantigen in multiple sclerosis (Salvetti et al., 1996). Immunizations with recombinant Hsp65, and Hsp65 rich *M. tuberculosis* in C57BL/6J mice induced atherogenesis indicating the involvement of Hsp65 in atherogenesis (George et al., 1999).

7. Hsps in vaccine development against TB

Hsps are among the proteins that are expressed at high level during the TB infection and are highly conserved. They could mediate the T cell sensitization required for the production of antibodies and can be used in the development of vaccine(s) against TB (Lussow et al., 1991). Inside the host, T cells are involved in activating macrophages and controlling the mycobacterial infection. Both the macrophage and mycobacterium synthesize heat shock proteins in order to facilitate their survival, and these Hsps possess potent immunogenicity (Munk & Kaufmann, 1991). The Hsp70 of *M. tuberculosis* has been shown to have anti-inflammatory properties and immunosuppressive role in a graft rejection system (Borges et al 2010). Hsp70 is recognized by human $CD4^+$ T-cells and it leads to the secretion of TNF, IL-6 and IL-1β (Asea et al., 2000). Mycobacterial Hsp70 can be used in subunit vaccine design since it contains a variety of T-cell epitopes (Oftung et al., 1994). Studies have been done to map the epitopes of Hsp70, so as to eliminate the autoimmune response in humans (Adams et al., 1993). A synthetic peptide, non-covalently bound to *M. tuberculosis* Hsp70 generated a very strong specific proliferative T-cell response in the spleen of mice (Roman & Moreno, 1996).

Hsp16 also induces T-cells to proliferate and secret cytokines, and therefore can be used as a potential subunit vaccine candidate (Agrewala & Wilkinson, 1999). A DNA vaccine combination expressing mycobacterial Hsp65 and IL-12 provided high degree of protection against TB (Okada et al., 2007). The vaccine was delivered by the hemagglutinating virus of Japan (HVJ)-envelope and liposome. This vaccine provided remarkable protection in mice and monkeys compared to the BCG vaccine, demonstrating the potential of Hsps to be used in vaccine development (Okada, 2006; Okada & Kita; Okada et al., 2007; Okada et al., 2009). A prime-boost strategy was investigated in cattle, using a combination of three DNA vaccines coding for Hsp65, Hsp70, and another mycobacterial protein Apa for priming, followed by a boost with BCG prior to experimental challenge with virulent *M. bovis* (Skinner et al., 2003).

Hsp65 as an antigen can confer protection equal to that from live BCG vaccine (Silva, 1999). The mycobacterial Hsp65 and Hsp70 acted as carrier molecules in mice previously primed with *M. tuberculosis* and showed high and long-lasting titers of IgG (Barrios et al., 1992; Perraut et al., 1993). The mycobacterial Hsp65 conjugated to peptides or oligosaccharides in the absence of adjuvants, induced antibodies which cross-reacted well with Hsp homologues from other prokaryotes, but weakly with the human Hsp homologue (Barrios et al., 1994). The PBMCs and T-cell lines from *M. leprae* and *M. bovis* BCG vaccinated subjects showed proliferation in response to Hsp18 and Hsp65 of *M. leprae*, Hsp65 of *M. bovis* BCG, and the Hsp70 of *M. tuberculosis* (Mustafa et al., 1993). The response of T cells to these Hsps makes them eligible for their application in the next generation of subunit vaccines (Mustafa et al., 1993).

8. Hsps in TB diagnosis

In high-TB incidence countries, TB control relies on diagnosis which is mainly based on clinical symptoms or laboratory diagnosis using sputum smear microscopy. TB smear microscopy is highly insensitive for HIV-co-infected individuals and for children due to the reduced pulmonary bacillary loads in these patients. TB diagnosis by smear microscopy is usually further confirmed by culture. However, this requires extended incubation times and is significantly more expensive than smears, requiring specialized equipment and highly trained personnel (Parsons et al., 2004; Storla et al., 2008). Thus, there is a basic need for the development of fast and inexpensive ways of TB diagnosis.

Using recombinant DNA techniques, synthetic peptides, antigen-specific antibodies and T cells, several major antigens of *M. tuberculosis* have been identified which include hsp60, hsp70, Ag85, ESAT-6 and CFP10 (Mustafa, 2001). In addition, Hsp65, Hsp71, 14-kDa Hsp and GroE proteins can play an important role in the diagnosis of TB. The identification of these markers can contribute to the clinical diagnosis of TB and may also provide additional insight into the pathogenesis of TB (Kashyap et al., 2010). sHsp18 has been shown to be a major immunodominant antigen of *M. leprae* (Lini et al., 2008). Hsp65 has been shown to be an attractive marker for TB (Bothamley et al., 1992; Haldar et al., 2010; Lee et al., 1994; Rambukkana et al., 1991). The 65 kDa heat shock protein is detected even in the cerebrospinal fluid of tuberculous meningitis patients, indicating its potential use as a diagnostic marker for tuberculous meningitis (Mudaliar et al., 2006). In case of TB ascites, the ascitic fluid has shown the presence of Hsp65, Hsp71 and Hsp14 as very useful diagnostic markers (Kashyap et al., 2010). A multiplex PCR against Hsp65 gene coding for 65 kDa antigen for early detection has been tested. The technique was able to distinguish between strains of the *M. tuberculosis* complex and non-tuberculous mycobacteria (Bhattacharya et al., 2003).

The response to recombinant 10-kDa heat shock protein of *M. leprae* was evaluated by indirect ELISA in sera from leprosy patients, household contacts, tuberculosis patients and healthy controls. However, this test seems to have a low sensitivity and specificity for leprosy detection and tuberculosis patients sera cross-reacted with *M. leprae* antigen as well (Rojas et al., 1997).

9. Conclusion

Treatment for *M. tuberculosis* has to be lengthy, since populations of this bacillus differ in metabolic activity. In addition, the treatment has to consist of a variety of drugs, since spontaneous chromosome mutations can give rise to drug resistance. As heat shock proteins are involved in crucial housekeeping activity, their inactivation may be lethal for the cells. Despite the impressive progress in the understanding of structure and function of Hsps, the biological significance of these proteins in the survival of bacteria inside macrophage is still not completely clear. These proteins appear to be playing an important role in protecting the bacteria from the environment. Further understanding of Hsps is required for the development of new anti-tuberculosis drugs and vaccines.

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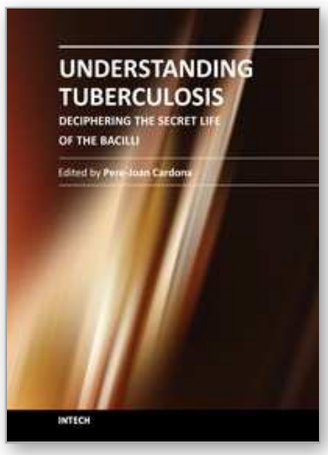

Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli

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Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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