CYTOKINES AND THE REGULATION OF HEAT SHOCK PROTEINS IN SYSTEMIC LUPUS ERYTHEMATOSUS

by

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Institute of Child Health,
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2001
To my family
- for all their love and support
Abstract

Previous studies have reported elevated levels of the 90 kDa heat shock protein (Hsp 90) and autoantibodies to Hsp 90 in a subset of patients with systemic lupus erythematosus (SLE). However, the mechanisms leading to the overexpression of Hsp 90 in SLE and a link between this event and the induction of Hsp 90 autoantibodies, have remained unclear. Recent studies have shown that the cytokine IL-6, which is elevated in SLE, activates Hsp 90 gene expression in cultured cells via specific transcription factors, that include STAT-3.

In view of the known role of STAT proteins in signalling pathways of the cytokine IL-10, the effect of IL-10 on Hsp 90 levels in different cells was investigated. IL-10 enhanced the expression of Hsp 90 in cultured cells. In reporter gene assays, IL-10 activated both the Hsp 90α and Hsp 90β promoters directly. Activation of the Hsp 90β promoter was mediated by a short specific region of the promoter which contains a binding site for STAT-3.

Cytokines and Hsps, were quantified in individual blood samples from patients with SLE. Levels of Hsp 90 positively correlated with levels of IL-6 but not IL-10, in patients with SLE. Furthermore, this correlation was enhanced in patients who overexpressed Hsp 90 (a subset of patients associated with HLA DR3 negativity).

A link between Hsp 90 overexpression and the development of Hsp 90 autoantibodies was also investigated. Antibodies to Hsps in patients with SLE were quantified. Overexpression of Hsp 90 in SLE correlated with raised levels of antibodies to Hsp 90 in the same patients. Furthermore, antibodies to Hsp 90 were associated with active general disease features of SLE.

These results support a role for IL-6 in the overexpression of Hsp 90, leading to raised levels of antibodies to Hsp 90 and active disease in SLE.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>a.u.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E.coli</td>
<td><em>Esherichia coli</em></td>
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<tr>
<td>g</td>
<td>grammes</td>
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<td>hr</td>
<td>hours</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>kDa</td>
<td>kilodaltons</td>
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<tr>
<td>M</td>
<td>molar</td>
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<td>mM</td>
<td>millimolar</td>
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<td>mn</td>
<td>minutes</td>
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<tr>
<td>mg</td>
<td>milligrammes</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>n</td>
<td>number of subjects</td>
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<tr>
<td>ng</td>
<td>nannogrammes</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>pg</td>
<td>picogrammes</td>
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<tr>
<td>p</td>
<td>P value</td>
</tr>
<tr>
<td>r</td>
<td>correlation coefficient</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>w/v</td>
<td>weight to volume ratio</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>μg</td>
<td>microgrammes</td>
</tr>
<tr>
<td>μl</td>
<td>microlitres</td>
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List of Publications


Ripley, B. J., Stephanou, A., Isenberg, D. A., and Latchman, D. S. (2001). Elevated levels of the 90kDa heat shock protein (hsp90) in SLE correlate with levels of IL-6 and autoantibodies to hsp90. (accepted for publication in Journal of Autoimmunity)
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Chapter 1

Introduction
Introduction

Section 1.1: The Heat Shock Proteins

Introduction

Since the discovery of heat shock proteins nearly 40 years ago, it is now clear that this group of proteins perform critical cellular roles under physiological and stressful conditions. Studies which have focussed on the importance of these proteins to cell survival, have been extended in recent years to suggest a role for Hsps in the pathogenesis of a number of human autoimmune diseases.

The heat shock response was first reported in 1962 by Ritossa. In this study, exposure of the giant salivary gland chromosomes of the fruitfly *Drosophila buksii* to elevated temperature, resulted in areas of swelling known as puffs. These puffs, which could also be induced by sodium salicylate and dinitrophenol, were presumed to occur as a result of specific and rapid transcriptional activity (Ritossa, 1962). In 1974, the phenomenon of *Drosophila* chromosome puffing was further characterised by Tissières. Following heat shock or treatment with the hormone ecdysone, changes in chromosome puffing patterns were observed to correlate with temporal increases in specific polypeptide synthesis, against a background of general protein synthesis repression (Tissieres et al., 1974). The novel set of proteins described in this study are now collectively referred to as heat shock proteins (Hsps). Following observations with *Drosophila*, Hsps have been shown to be expressed in prokaryotes, eukaryotes and plant cells. Importantly, in all cell systems studied, levels of Hsps are increased not only by elevated temperature (heat shock), but also by a variety of other environmental stresses including viral/microbial infections, heavy metals, toxins and amino acid analogues, as well as by non-stressful conditions including cytokines and growth factors (Lindquist, 1986), (Polla, 1988).

A critical role for Hsps in cell survival following exposure to various stressful conditions, appears to be an extension of their role in normal cells during non-stressful conditions. Thus, some Hsps are expressed at constitutively high levels in cells and have roles in normal cell growth and development (Bond and Schlesinger, 1987), while other Hsps are present at much lower levels in cells and their synthesis
is elevated in response to stressful conditions. A common link between these studies, is an association of Hsp function with the accumulation of unfolded or malfolded proteins (Gething and Sambrook, 1992).

**Heat Shock Protein Families And Biological Functions**

The importance of Hsps to cell survival is evidenced by a high degree of homology at the genomic level observed for Hsps between species, from bacteria to mammals (Lindquist and Craig, 1988). Thus, shared structural and cellular functions of Hsps are observed between highly diverse species and these have been conserved throughout evolution. The earliest studies of Hsp cell biology highlighted their increased synthesis in response to heat and other stresses. Studies over the last decade however, have shifted the focus of attention to understanding the role of Hsps as molecular chaperones, assisting the processes of protein synthesis, protein folding and degradation, as well as protein translocation within the cell.

Many Hsps and their associated co-chaperones are constitutively expressed in cells. A number of multi-gene Hsp families are recognised and individual genes within families are observed to differ with respect to their sequence and pattern of expression, as well as their function within the cell and their sub-cellular localisation. Heat shock proteins are classified into families according to their approximate molecular weights (in kilodaltons). In mammals, the major Hsp families are designated Hsp 90 (approximately 90 kDa Hsps), Hsp 70 (approximately 70 kDa Hsps), Hsp 60 (approximately 60 kDa Hsps), small Hsps (22-32 kDa Hsps) and ubiquitin (approximately 8 kDa). In this section, the classification, structure and cellular roles of these Hsp families are outlined and a summary of the major roles of mammalian Hsps is provided in Table 1.1.1.
<table>
<thead>
<tr>
<th>Family</th>
<th>Members</th>
<th>Characteristics / Biological Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hsp 90</strong></td>
<td>Hsp 90α</td>
<td>Hsp 90α more heat inducible than Hsp 90β.</td>
</tr>
<tr>
<td></td>
<td>Hsp 90β</td>
<td>In mammals it is the most abundant cytoplasmic Hsp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Together, both isoforms constitute 1-2% of total cytoplasmic protein. Regulatory interactions with signalling proteins. Involved in cell growth and development and antigen processing/presentation. Hsp 90 Binds ATP, inherent ATPase activity.</td>
</tr>
<tr>
<td></td>
<td>GRP94</td>
<td>Glucose regulated protein (GRP). Located at plasma/nuclear membranes and within Golgi.</td>
</tr>
<tr>
<td><strong>Hsp 70</strong></td>
<td>Hsp 72</td>
<td>Typically stress-induced.</td>
</tr>
<tr>
<td></td>
<td>Hsp 73</td>
<td>Largely constitutively expressed.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Located in cytoplasm and nucleus. Roles in protein biosynthesis (transport and folding) and cell cycle control. Bind ATP, ATPase activity.</td>
</tr>
<tr>
<td></td>
<td>GRP78</td>
<td>Present in ER and lysosomes. Expressed at high levels in secretory cells. Roles in protein import, modification and assembly.</td>
</tr>
<tr>
<td></td>
<td>GRP75</td>
<td>Present in mitochondria. Similar role to GRP78.</td>
</tr>
<tr>
<td><strong>Hsp 60</strong></td>
<td></td>
<td>Present in mitochondria as large oligomers. Roles in protein assembly and cellular respiration. Binds ATP, weak ATPase activity.</td>
</tr>
<tr>
<td><strong>Small Hsps</strong></td>
<td>Hsp 27</td>
<td>Oligomeric structure which re-distributes inside or around nucleus upon heat shock. Role in growth and differentiation in normal and cancerous cells associated with involvement in actin filament dynamics.</td>
</tr>
<tr>
<td></td>
<td>Ubiquitin</td>
<td>Located in cytoplasm and nucleus. Role in conjugation to stress-damaged proteins, facilitating degradation by enzymes of an ubiquitin-dependent pathway.</td>
</tr>
</tbody>
</table>
Introduction

The Hsp 90 Family

The Hsp 90 family is a group of proteins localised within the cell cytoplasm of eubacteria, yeast and multicellular organisms. Homologues have been characterised and are found in the endoplasmic reticulum (ER) of eukaryotes (known as Grp94) and in chloroplasts (known as cpHsp82). Hsp 90 is a highly abundant cytoplasmic protein and is estimated to comprise 1-2% of total soluble protein in eukaryotic cells (Welch and Feramisco, 1982) and its expression in yeast (Borkovich et al., 1989) and Drosophila melanogaster (Cutforth and Rubin, 1994), is essential. Two isoforms of Hsp 90, which are encoded by separate genes termed Hsp 90α and Hsp 90β, are found in eukaryotic cells and are differentially expressed. Both isoforms share a high degree of amino acid sequence identity to each other; approximately 85% sequence identity in humans (Hickey et al., 1989) and approximately 90% sequence identity in yeast (Borkovich et al., 1989). A further cytoplasmic Hsp 90 family member, termed TRAP1, is found in higher eukaryotes and is present at much lower levels compared to both Hsp 90α and Hsp 90β (Felts et al., 2000).

Studies of Hsp 90 have examined the native protein's modular structure. Two highly conserved regions, an N-terminal domain (approximately 25 kDa) and a C-terminal region (approximately 55 kDa), are connected by a highly charged linker, which can be of variable length between species and isoforms. The complete absence of this charged region in some prokaryotic and mammalian Hsp 90 family members (see Figure 1.1.1a), would suggest it is not important for Hsp 90 function. Hsp 90 exists predominantly as a dimer in vivo and this occurs through the anti-parallel association of C-terminal regions of each monomer (shown in Figure 1.1.1b). The native forms of Hsp 90 have been studied in the mouse, using non-denaturing gel electrophoresis (Minami et al., 1991) and in the rat liver cytosol, using isoform-specific monoclonal antibodies (Nemoto and Sato, 1998). These studies showed that the purified Hsp 90 existed predominantly as a dimer, but a considerable amount of monomer was also detected. In addition, the dimeric forms were found to consist of alpha/alpha (α/α) and beta/beta (β/β) homodimers and to a lesser extent alpha/beta (α/β) heterodimers. The monomeric form was found to consist mainly of the beta (β)
Figure 1.1.1 The Domain Structure of Hsp 90 and a Schematic Representation of the Native Dimeric Form, adapted from Buchner, (1999)

(a) Domain structure of Hsp 90. The charged linker is shown in black and is much reduced in length (shown as a thin black line) in some eukaryotic and prokaryotic Hsp 90 species. Highly conserved regions are shown in light and dark grey.

(b) Conformational flexibility of the native dimeric form of Hsp 90. In response to heat shock or binding of ATP (A), an open structure with N-terminal domains (N) pointing in opposite directions, changes to one which is closed and toroidal in shape.
**Introduction**

isoform. In mammals, both of the Hsp 90 isoforms have been shown to exist as oligomeric structures in the cytoplasm under both physiological and stressful cell conditions (Nemoto and Sato, 1998). Crystal structures for N-terminal domains of human (Prodromou et al., 1997a) and yeast Hsp 90 (Prodromou et al., 1997b) have been reported. These studies show the N-terminal domain to contain binding sites for nucleotide, involved in ATP binding and hydrolysis (Prodromou et al., 1997a) and the anti-tumour drug geldanamycin (which inhibits Hsp 90 function). Polypeptide binding sites have been shown to exist in both the N-terminal and C-terminal domains, with each displaying a different substrate preference (Young et al., 1997), (Scheibel et al., 1998). The overall structure of the N-terminal domain consists of an eight-stranded anti-parallel β-sheet and nine α-helices which together form an α-β sandwich. In the yeast Hsp 90 dimer, the opposing faces of the β-sheets have been suggested to form a potential peptide-binding cleft. Thus, the N-terminal domain may act as a molecular “clamp” for the binding of Hsp 90 substrates (Prodromou et al., 1997b). To date however, no structures for either a C-terminal domain or an intact Hsp 90 molecule, have been determined. Antibody and electron microscopy studies have detected conformational flexibility in the native dimeric structure. As shown in Figure 1.1.1b, heat shock or binding of ATP causes specific structural transformations, in which the relative orientation of N-terminal domains of each monomer, switches from an open conformation to one which is closed and toroidal in shape (Maruya et al., 1999).

The abundance of Hsp 90 in eukaryotic cells and its elevated expression in response to various stimuli, suggest an important role for Hsp 90 during physiological and stressful conditions. Molecular chaperones are polypeptide-binding proteins which facilitate protein folding by shifting the equilibrium of the folding reaction towards a productive folded state and away from the aggregated state (Hartl, 1996), (Bukau and Horwich, 1998). Hsp chaperones, including Hsp 90 and Hsp 70, are unable to perform such a role on their own, but function within multi-component complexes involving co-chaperones and their co-factors. In comparison with other Hsp families (particularly Hsp 70), an understanding of the function of Hsp 90 *in vivo*
remains obscure. To date, the majority of studies on Hsp 90, have revealed and focussed on Hsp 90 roles in the conformational regulation of proteins involved in signal transduction and cell-cycle control. These observations contrast with those of other Hsp families, such as Hsp 70 and Hsp 60, which in comparison with Hsp 90, display far less specificity for the type of protein with which they interact. However, the list of known Hsp 90 substrates is steadily growing (see Table 1.1.2) and these advances now suggest the Hsp 90 chaperone machinery to have a multifunctional role in protein folding in the cell and thus a more dynamic involvement than previously thought (Buchner, 1999). In particular, the recent observation (Panaretou et al., 1998) that Hsp 90 like other Hsp families, can function in an ATP-dependent manner (previously thought not to be the case), exemplifies the growing understanding of Hsp 90 function and the scope of possible roles for Hsp 90 as a molecular chaperone in the cell (discussed in Grenert et al., 1999).

Hsp 90 interacts with two classes of "classical" Hsp 90 "client" proteins, the steroid-hormone receptors and kinases and the list of other Hsp 90 substrates is gradually increasing. As shown in Table 1.1.2, proteins whose folding is known to be affected by Hsp 90 not only include transcription factors such as heat shock factor (Zou et al., 1998) and kinases such as casein kinase 2 (Miyata and Yahara, 1995), but also proteins with functions unrelated to these "classical clients", such as the microtubule cytoskeletal component tubulin (Gamier et al., 1998) and the enzyme nitric oxide synthase (Garcia-Cardena et al., 1998).

One of the most studied roles of Hsp 90 in protein folding within the cell, involves the interaction between Hsp 90 and the steroid hormone receptors (SHRs). The SHRs comprise three separate domains which are separately involved in hormone binding, DNA binding and transcriptional activation (Evans, 1988). In this respect SHRs may be considered as direct signal transducers. Thus, in the hormone-activated state, SHRs enhance gene transcription by binding to specific DNA sequences in target genes. Studies in the 1980s established the involvement of Hsp 90 in the conformational regulation of steroid hormone receptors and is reviewed in Pratt and Toft, (1997). More recently, a greater understanding of this process has been achieved through the identification of components of the Hsp 90 chaperone complex.
Table 1.1.2  Examples of Hsp 90 Client Proteins

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Client Protein</th>
</tr>
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<tbody>
<tr>
<td>Protein Kinases</td>
<td>PI-4 kinase</td>
</tr>
<tr>
<td></td>
<td>v-Src</td>
</tr>
<tr>
<td></td>
<td>Raf-1</td>
</tr>
<tr>
<td></td>
<td>Mek</td>
</tr>
<tr>
<td></td>
<td>elf-2α kinase</td>
</tr>
<tr>
<td></td>
<td>calcineurin</td>
</tr>
<tr>
<td></td>
<td>casein kinase 2</td>
</tr>
<tr>
<td></td>
<td>Ste11</td>
</tr>
<tr>
<td></td>
<td>Gcn2</td>
</tr>
<tr>
<td>Receptors/Transcription Factors</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td></td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td></td>
<td>Eosstrogen receptor</td>
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<tr>
<td></td>
<td>Androgen receptor</td>
</tr>
<tr>
<td></td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td></td>
<td>P53</td>
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<tr>
<td></td>
<td>Heat shock factor</td>
</tr>
<tr>
<td>Other</td>
<td>Insulin</td>
</tr>
<tr>
<td></td>
<td>Tubulin</td>
</tr>
<tr>
<td></td>
<td>Citrate synthase</td>
</tr>
<tr>
<td></td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td></td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td></td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td></td>
<td>Luciferase</td>
</tr>
<tr>
<td></td>
<td>Dihydrofolate reductase</td>
</tr>
</tbody>
</table>
Introduction

The conformational changes in SHRs which allow binding of hormone to the receptor to take place, involve the interaction of several Hsp family chaperone complexes (see Figure 1.1.2). The sequence of events which lead to a SHR in the hormone-activated state, begins with the interaction of the SHR with Hsp 70 and its co-factors. Following this, an intermediate complex forms through the interaction of an Hsp 90 chaperone complex. This appears to be facilitated via the scaffolding protein Hop, which has been demonstrated to be required for the interaction of both chaperone families (Frydman and Hohfeld, 1997). The formation of a mature complex, in which SHRs can bind hormone, requires the dissociation of Hsp 70 and its co-factors Hip and Hsp 40. The binding and hydrolysis of ATP at a site within the N-terminal domain of Hsp 90 (Prodromou et al., 1997a), allows a mammalian protein (p23) and a member of the immunophilin protein family, to bind the Hsp 90-SHR complex (Grenert et al., 1997). Finally, the SHR is released from the Hsp 90 complex, it is then able to bind hormone, dimerise and subsequently bind to DNA.

As shown in Table 1.1.2, Hsp 90 also interacts with transcription factors that are not steroid receptors, including heat shock factor (HSF), the p53 tumour suppressor protein, and the dioxin receptor. The tumour suppressor protein p53, is a transcription factor whose mutant form is often found in tumour cells and Hsp 90 has been observed to form a heterocomplex with mutant p53 (Stancato et al., 1993).

Members of the Src family of tyrosine kinases have been shown to be Hsp 90 clients, highlighting a role for Hsp 90 in signal transduction from receptors in the plasma membrane. For example, the oncoprotein pp60<sup>v-src</sup> (v-Src), a cytoplasmic membrane-associated tyrosine kinase, has been reported to interact with Hsp 90 and this was the first observed stable complex of a protein with Hsp 90 (Brugge, 1986). Following its synthesis in vertebrate cells and subsequent interaction with Hsp 90, v-Src is not functional as a kinase. Within the plasma membrane however, v-Src is active as a kinase and is no longer associated with Hsp 90. Genetic studies in yeast, involving mutations that lower the level of Hsp 90 (Xu and Lindquist, 1993), suggest that Hsp 90 both stabilises v-Src and suppresses its kinase activity during transit to the plasma membrane. The Hsp 90-dependence of v-Src and other Src family members including v-Yes and v-Fps, has not been observed for their normal cellular counterparts. However, studies in yeast have shown the activation of both v-Src and
The Hsp 90 chaperone complex involved in SHR activation. The SHR interacts sequentially with several chaperone complexes. Initially this involves Hsp 70 and cofactors. The scaffold protein HOP brings together the Hsp 90 chaperone machinery with Hsp 70 to form an intermediate complex (1). The Hsp 70 complex then dissociates (2) and p23 and one of the large immunophillins (IP), enter the complex (3). The SHR is released from this complex, it can then bind hormone, dimerise and bind DNA.
the normal cellular homologue c-Src, to be Hsp 90-dependent and thus these observations highlight the possibility that the activation of other normal cellular Src family members is also Hsp 90-dependent (Xu et al., 1999).

Some receptors, such as the insulin and epidermal growth factor (EGF) receptor, transduce their signal from the plasma membrane through activation of members of the mitogen-activated protein (MAP) kinase family of serine/threonine kinases. Components of this system, which have been shown to form stable complexes with Hsp 90, include Src, Raf and Mek (Pratt, 1997). Not suprisingly, these observations have highlighted the suitability of Hsp 90 as a candidate target for anti-cancer drug development. In agreement with this, the antibiotic geldanamycin (which displays tumouricidal activity), has been shown to function through the inhibition of Src-Hsp 90 heterocomplex formation (Whitesell et al., 1994).

**The Hsp 70 Family**

The Hsp 70 family consists of a group of highly conserved proteins which are present in prokaryotic and eukaryotic cells under normal growth conditions and, as for Hsp 90, their expression is increased in response to various stressful and non-stressful conditions. Members of this family have a variety of roles which relate to their location within the cell and their function as molecular chaperones. These include the processes of protein folding and degradation, translocation of proteins across organellar membranes and auto-regulation of the heat shock response (discussed later). In mammals (see Table 1.1.1), members of this family include Hsp 72, the major heat-inducible Hsp 70 (also referred to as Hsp 70 or Hsx 70), Hsp 73 which is largely constitutively expressed (also called Hsc 70), Grp 78 which is present in the endoplasmic reticulum (also known as immunoglobulin binding protein, BiP) and Grp 75 which is present in mitochondria. The structure of Hsp 70 is highly conserved from prokaryotes to mammals. Two domains are present, an N-terminal domain (approximately 40 kDa) contains an ATP-binding site (Flaherty et al., 1990) and a C-terminal domain (approximately 25 kDa) which is less conserved between species and functions in substrate binding.
Introduction

A number of studies have suggested a role for Hsp 70 in the de novo folding of proteins and are reviewed in Feldman and Frydman, (2000). The process of folding a newly translated polypeptide requires its immediate and subsequent sequential interaction with different chaperone complexes. This is necessary to prevent various processes occurring such as protein aggregation, which inhibit the formation of mature, appropriately folded and functional proteins, reviewed in Gething and Sambrook, (1992). The process of protein translation is understood to proceed within different time-scales from prokaryotes (seconds) to eukaryotes (several minutes). Thus, particularly in eukaryotic cells, it is conceivable that the folding of a polypeptide nascent chain may begin prematurely during the translation process and critically, before completion of synthesis of a complete folding domain (a structurally independent unit). A similar problem occurs during the translocation of proteins across the membranes of mitochondria, chloroplasts and endoplasmic reticulum, where an extended polypeptide conformation rather than a stably folded protein, is required for protein transport.

Hsp 70 family chaperones in cooperation with co-chaperones of the Hsp 40/DnaJ family, bind small linear polypeptides with a net hydrophobic character (exposed hydrophobic regions are a characteristic feature of unfolded proteins) leading to polypeptide stabilisation in an extended conformation which prevents aggregation (Gething and Sambrook, 1992). Further studies have highlighted a role for Hsp 70 in the folding of nascent polypeptides. For example, yeast strains with a loss of Hsp 70 function, show reduced translational efficiency (Nelson et al., 1992). Furthermore, cytoplasmic Hsp 70 is observed to associate with ribosome-bound nascent chains in eukaryotic cells (Nelson et al., 1992), (Frydman et al., 1994). Hsp 40 and DnaJ (a constitutively expressed 40 kDa chaperone) family chaperone proteins are understood to be involved in the process of Hsp 70 recruitment to nascent polypeptide chains, reviewed in Bukau and Horwich, (1998), while Hsp 70s of the mitochondria and endoplasmic reticulum are observed to bind to translocating polypeptides (Manning-Krieg et al., 1991), (Brodsky and Schekman, 1993). Following translocation, mitochondrial and endoplasmic reticulum (ER) Hsp 70s are required for the subsequent folding of polypeptides. However, in many cases the Hsp 70 chaperones alone or in conjunction with the Hsp 90 chaperone complex, are
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insufficient for the folding of polypeptides to their native state. More complex protein folding is aided by the transfer of these polypeptides to chaperonin family members such as Hsp 60.

The Hsp 60 Family

Members of this family (also known as chaperonin 60) are among the most abundant of cellular proteins found in bacteria (known as GroEL in *E.coli*) and are also present in the mitochondria and chloroplast of eukaryotes. A structurally related chaperonin family has been identified, the TriC family. This is found in the cytoplasm of most eukaryotes, including plants, yeast, *Drosophila*, archaebacterial cells, and appears to fulfil the functions of mitochondrial Hsp 60. Structurally, the Hsp 60s form large oligomeric complexes comprising 60 kDa subunits, which are arranged as two stacked heptameric rings forming a central cavity (Hendrix, 1979), (Hohn et al., 1979). Although members of the Hsp 60 family are induced under conditions which can cause protein denaturation (Hightower, 1991), the Hsp 60s are constitutively expressed and are essential for growth.

Members of the Hsp 60 family cooperate with a smaller stress-inducible protein, Hsp 10 (also known as chaperonin 10) in the process of protein folding. In *E.coli*, the Hsp 60 (GroEL) and the Hsp 10 (GroES), are encoded in a single operon (Tilly et al., 1981), (Chandrasekhar et al., 1986). Chaperonin proteins, which include the Hsp 60 family, function to assist *de novo* protein folding by sequestering proteins in the early stages of folding and providing a protective environment (the chaperonin cavity) in which to fold. The majority of client proteins for Hsp 60s tend to be of medium size (25-60 kDa). Small proteins, which generally have rapid folding kinetics, appear to require minimal assistance in preventing malfolding. Furthermore, a sub-population of medium size proteins would appear to require assistance in folding, in addition to that provided by Hsp 70 and co-chaperones. For example, the enzyme dihydrofolate reductase (DHFR), folds spontaneously *in vitro*, but requires several chaperone families, including mitochondrial Hsp 60 (Ostermann et al., 1989), for appropriate folding *in vivo*.  

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Subsequent studies have shown Hsp 60 to be involved in the assembly of various oligomeric complexes. In yeast defective for the gene encoding mitochondrial Hsp 60, chaperonin function has been shown to be essential for the assembly of oligomeric enzyme complexes (Cheng et al., 1990) and in prokaryotes, GroEL and GroES are responsible for the folding of subunits of the enzyme ornithine transcarbamylase (OTC) (Zheng et al., 1993). A role for Hsp 60 in protein folding under conditions of heat stress conditions is supported by the observation that the exposure of mitochondria to heat stress, leads to the association of a variety of polypeptides with mitochondrial Hsp 60 (Martin et al., 1992). Under such conditions, functional Hsp 60 has been demonstrated to be required to prevent thermal inactivation of the enzyme dihydrofolate reductase (DHFR) imported into the mitochondrion.

In considering the differential binding capabilities of Hsp 70 and Hsp 60, these observations would suggest that the folding of newly synthesised polypeptides follows complex pathways. Thus, it is likely that a mechanism of sequential action of Hsp 70 and Hsp 60 chaperones is involved in protein folding. Moreover, the identification of the structurally related TriC chaperone family, would suggest this mechanism could also be relevant to the eukaryotic cytosol.

A major interest of Hsp 60, is its possible role in the development of autoimmunity in humans. As discussed later (see Section 1.2), a number of studies have focussed particularly on the mycobacterial Hsp 60 homologue and Hsp immune autoreactivity in various inflammatory disease states such as arthritis.

Small Hsps and Ubiquitin

The family of small Hsps (sHsps) are comparatively less conserved among species than the high molecular weight Hsp families such as Hsp 90 and Hsp 70. Members of this family have molecular weights up to 40 kDa and are characterised by a conserved region, referred to as the α-crystallin domain. This domain is located in the C-terminal region of all sHsps (Kim et al., 1998) and in Drosophila and mammals, it consists of between 80 and 100 amino acids (Ingolia and Craig, 1982). In contrast, the N-terminus of sHsps is variable in both sequence and length. Studies
on sHsp expression have shown that sHsp genes are highly inducible in response to heat and other stresses. Differences in the stress-induced expression of the sHsps compared to other Hsp families have been observed in *Drosophila*, where sHsps tend to be expressed at temperatures lower than those required for Hsp 70 induction (Yost et al., 1990).

Constitutive expression of sHsps in unstressed cells has been well documented in *Drosophila* and appears to follow both a tissue and stage-specific pattern of expression. In mammals, Hsp 27 is constitutively expressed at low levels in various cell-lines, including human HeLa, monkey Cos and mouse Swiss-3T3 cells. Hsp 27 has also been observed to accumulate in the absence of stress within several tissues, including spinal cord neurons and Purkinje cells, during murine development (Gemold, 1993). In human and monkey cells, Hsp 27 expressed constitutively in the cytoplasm is observed, like αβ-crystallin, to have a peri-nuclear localisation (Arrigo et al., 1988). In contrast, Hsp 25 of chicken, is found to be diffusely distributed in the cytoplasm (Collier and Schlesinger, 1986). Studies on the localisation of sHsps within the cell, have highlighted distribution patterns which are a consequence of either stressful or non-stressful cellular conditions. For example, heat shock or other forms of cellular stress leads to the re-distribution of Hsp 27 (Arrigo et al., 1988) and αβ-crystallin (Klemenz et al., 1991), inside or around the nucleus. Subsequently, following cellular recovery from heat shock, sHsps are gradually re-distributed in the cytoplasm (Arrigo et al., 1980). All sHsps studied to date have been shown to possess oligomeric structures. During heat-shock, the size of Hsp 27 oligomers increase, resulting in a super-aggregated structure which re-distributes inside the nucleus (Arrigo et al., 1988). Furthermore, these observations do not pertain to Hsp 27 or other sHsps, in thermotolerant cells exposed to heat-shock (Arrigo and Welch, 1987).

Ubiquitin is a 7-8 kDa protein of 76 amino acids, which is highly conserved between species (Lindquist, 1986). Its expression within the cell is constitutive, but also highly inducible in response to cellular stresses such as elevated temperature (Schlesinger, 1990). Ubiquitin is involved in a multienzyme system responsible for the degradation of short-lived, misfolded or damaged proteins (Peters et al., 1998). Protein degradation is mediated by the 26S proteosome, a large ATP-dependent multicatalytic protease complex. The ubiquitin-proteosome pathway represents a
major cellular non-lysosomal pathway of proteolysis. Proteins are usually targetted for proteosome-mediated degradation, by the covalent attachment of multiple ubiquitin molecules (a process termed polyubiquitination) via terminal glycine residues of ubiquitin to other proteins (Lindquist and Craig, 1988), (Peters et al., 1998).

More recently, a role for ubiquitin in the downregulation of signal transducing receptors has been established. Binding of growth hormone to its receptor leads to receptor dimerisation, signal transduction and an increase in receptor ubiquitination. Ligand-induced endocytosis of the mammalian growth hormone receptor (GHR), occurs in a manner dependent on a functional ubiquitin conjugating system (Strous et al., 1996). Further studies have identified a ubiquitin conjugation motif within the cytoplasmic tail of the GHR. This is required for ligand-induced internalisation of the GHR, leading to degradation of the exoplasmic and cytoplasmic portions of the receptor (Govers et al., 1999). Thus, ubiquitin may be considered to have a role in the selective degradation of non-functional/malformed proteins as well as regulatory roles in a variety of receptor-associated cellular processes and is reviewed in van Kerkhof et al., (2000).

**Conditions that Induce the Expression of Heat Shock Proteins**

The term heat shock response clearly has a historical origin. Subsequent studies have shown that the conditions involved in inducing the expression of Hsps within cells are remarkably diverse. (see Figure 1.1.3). Thus, these conditions may be broadly categorised into three different classes: (1) environmental stress, including heat shock and exposure of cells to heavy metals, (2) cellular pathophysiology, including viral and bacterial infection and (3) non-stress conditions, including cytokines, cell cycle control and cellular growth and differentiation.
Figure 1.1.3 shows three broadly categorised classes of conditions which result in the elevated expression of Hsps. Also shown is the mechanism by which environmental stresses such as heat shock, induce Hsp expression via the activation (a) of heat shock factor (HSF) and its subsequent translocation into the nucleus where it binds to heat shock elements in the promoters of Hsp genes.
Introduction

Regulation of Heat Shock Protein Expression

The diversity of roles observed for different Hsp families within the cell and their increased expression in response to various stimuli (Figure 1.1.3), would suggest the process of Hsp gene expression to be both highly versatile and carefully regulated. The genes encoding Hsps are highly conserved and the promoter regions of Hsp genes contain multiple sites for regulating constitutive and inducible gene expression.

Transcription factors are proteins which bind to specific sequences in the promoter regions of their target genes, leading to activation or suppression of gene transcription. The induction of Hsps in response to various cellular stress conditions, is mediated by a group of transcription factors termed heat shock factors (HSFs), which bind to heat shock elements (HSEs) in the promoters of Hsps (Morimoto, 1993), (Lis and Wu, 1993). However, although HSEs are a distinguishing feature of Hsp family genes, members of the Hsp 90 and Hsp 70 families include glucose-regulated Hsp members (see Table 1.1.1) which are not heat-inducible. The cloning of HSF genes has revealed a multi-gene family and to date, three HSFs (HSF1, 2, 3) have been cloned from different organisms, including yeast, mouse and humans and a further family member, HSF4, has been identified in humans (Nakai et al., 1997). Subsequent studies have led to both structural and functional characterisation of these HSF family members.

Common to all HSFs is an N-terminal-situated DNA binding domain of approximately 100 amino acids, which contains a helix-turn-helix fold, composed of $\alpha$ (alpha)-helices 1, 2 and 3, where $\alpha$-helix 3 is the DNA recognition motif that docks within the major groove of the DNA helix (Harrison et al., 1994). A hydrophobic motif which is involved in the oligomerisation of HSF prior to DNA binding, is also N-terminal-situated. A comparison of the sequences of the vertebrate HSFs reveals
that within a single species, HSF family members are approximately 40% identical at the amino acid level. This is primarily due to the conserved sequences of the DNA-binding and oligomerisation domains and is discussed in Cotto and Morimoto, (1999). The C-terminal region is less conserved in sequence and contains a transcriptional activation domain.

Only HSFl has been shown to be involved in the heat induced activation of Hsp genes and also functions in response to other stresses, including heavy metals and amino acid analogues (Sarge et al., 1993). In contrast, a role for other HSF members has been less well characterised, although these may be involved in Hsp regulation in unstressed cells in response to a variety of processes including cell differentiation and development (Morimoto et al., 1992), (Morimoto, 1993). For example, HSF2 mRNA in mouse testis is subject to both developmental and cell-stage, type regulation (Sarge et al., 1994).

Under normal cellular conditions, HSFl in the cytoplasm is maintained as a latent monomeric form which is unable to bind DNA. Activation of HSFl in response to thermal stress (heat shock), leads to the formation of homotrimers which can subsequently bind to HSEs within the promoter region of Hsp genes (Wu, 1995). Following a period of intermediate (non-extreme) heat shock, the activation of HSFl is rapid (minutes) but on returning to normal temperature, HSFl reverts to its non-DNA binding form (Abravaya et al., 1991a). Heat-induced phosphorylation of HSFl correlates with increased HSFl transcriptional activity, however phosphorylation is not essential for DNA-binding (Sorger and Pelham, 1988).

Studies on the transcriptional activation of Hsp genes by HSFl in both yeast and mammalian cells, suggest an autoregulatory role for Hsps. For example, mutations that decrease Hsp 70 synthesis (Werner-Washburne et al., 1987) or Hsp 90 synthesis (Duina et al., 1998), lead to increased Hsp synthesis and cellular tolerance to heat shock. Thus, it is possible that the dissociation of HSFl from Hsp complexes
Introduction

during heat shock, facilitates the activation of HSF1 and leads to increased Hsp synthesis in the cell.

The realisation that cytokines (see Figure 1.1.3) interacting with their counterpart cell membrane receptor, can also provide a stimulus for activating Hsp gene expression, has further increased the scope of Hsp gene regulation. The involvement of cytokines in Hsp gene regulation and in particular the regulation of the Hsp 90 gene, is discussed below.

Cytokines

Cytokines are a diverse group of small proteins (typically 15-30 kDa). They are secreted by different cell types in a number of tissues. The physiological roles of cytokines are largely concerned with the coordination of processes between different cell types or with the cellular response to environmental stresses. Consequently, their lifetime in the bloodstream or other extracellular fluids is short, suggesting limited and regulated biological activity. Cytokines influence the phenotype of their target cells by binding to a specific counterpart cell-surface receptor. However, the intracellular signalling pathways utilised by cytokines to activate their target genes, are largely to be characterised. For a comprehensive outline of the biological roles of cytokines, see Roitt., (1998).

The interleukins are a class of cytokine which are produced by, or act upon, cells of the immune system. The identification and characterisation of new interleukins is continuing, however of particular interest to this dissertation (see Section 1.5 of this chapter) are the cytokines interleukin-10 and interleukin-6, which are described below.
Introduction

**Interleukin-10**

Interleukin-10 (IL-10) is a multifunctional cytokine produced by B cells, T helper cells, monocyte/macrophages and keratinocytes. Human and murine IL-10 inhibit cytokine synthesis by activated T cells (Fiorentino et al., 1989; 1991), (de Waal Malefyt et al., 1991), NK cells (Hsu et al., 1992) and monocytes/macrophages (Bogdan et al., 1991), (de Waal Malefyt et al., 1991), (Fiorentino et al., 1991). Thus, IL-10 may be considered to have largely anti-inflammatory properties on cells of the immune system, for a review see Moore et al., (1993). These biological roles are mediated by IL-10 interacting with a counterpart cell surface receptor (see Figure 1.1.4). Characterisation of the human IL-10 receptor began in 1994 with the cloning of the human IL-10 receptor-binding chain (IL-10R1). This receptor was defined structurally as possessing a single transmembrane domain and as a member of the class II cytokine receptor family (Liu et al., 1994). Further studies have identified human CRFB4, a receptor belonging to the same cytokine family, as forming a functional constituent of the IL-10 receptor complex and it is now referred to as IL-10R2. Members of the class II cytokine receptor family, which include the interferon receptors, have been shown to mediate intracellular signalling via the Jak/STAT signalling pathway (Finbloom and Winestock, 1995) and is discussed later in this section.

**Interleukin-6**

Interleukin-6 (IL-6) is a multifunctional cytokine with pivotal roles in regulating the functions of lymphoid and myeloid cells as part of the normal immune response (Kishimoto and Hirano, 1988) as well as the production of acute phase proteins within the liver (Andus et al., 1987). The biological properties of IL-6 may
Figure 1.1.4 illustrates the structure of the IL-10 receptor following binding of IL-10 and receptor dimerisation. Phosphorylation and activation of the receptor-associated Jaks, leads to the recruitment and activation of STAT transcription factors, which subsequently enter the nucleus and bind to the promoters of their target genes.
be classified as pro-inflammatory through the promotion of differentiation and activation of T cells and macrophages.

IL-6 mediates its pleiotropic effects through an interaction with a specific counterpart receptor. An 80 kDa single polypeptide chain, termed gp80, binds IL-6 within a C-terminal extracellular region of the receptor containing a type III fibronectin domain (see Figure 1.1.5). Binding of IL-6 to gp80 subsequently promotes the association of this receptor with a non-ligand binding chain termed gp130 (Taga et al., 1989) which mediates IL-6 intracellular signalling. A soluble form of the gp80 receptor has also been identified and is generated either by proteolytic cleavage of the cognate receptor (PC-sIL-6R) or by deletion of the transmembrane domain by differential mRNA splicing (DS-sIL-6R) (Jones et al., 1998). In mice, serum soluble gp80 has been demonstrated to mediate IL-6 functions via cells expressing gp130 (Suzuki et al., 1993). The association of gp130 with IL-6 and other IL-6-type cytokine receptors, such as leukaemia inhibitory factor (LIF), which lack intrinsic transducing activity, therefore provides a common signalling mechanism for these receptor types.

Although the pro-inflammatory effects of IL-6 contrast with the biological roles of IL-10, both cytokines share similar signalling mechanisms for activating their target genes.

**IL-10 and IL-6 Activate the STAT Family of Transcription Factors**

The interaction of STATs (signal transducers and activators of transcription) with cytokine receptors which utilise the Jak/STAT signalling pathway, is mediated via SH2 (src homology 2) domains within the STAT protein, with specificity for a phosphotyrosine sequence motif within the intracellular domain of the cytokine receptor (Darnell, 1997). Tyrosine residues within the receptor-bound STAT
Figure 1.1.5  IL-6 Receptor Signalling via the JAK/STAT Pathway

Figure 1.1.5. Structure of the IL-6 receptor. Also shown is the sequence of events leading to the activation of the IL-6-activated transcription factor STAT 3, its translocation into the nucleus and binding to a promoter in a target gene.
molecule then become phosphorylated, leading to the formation of homo- or hetero-STAT dimers utilising reciprocal SH2 domains. STAT dimers are then translocated into the nucleus where binding to specific elements within the promoters of genes, leads to activation of gene transcription (Ihle, 1996).

The Janus family of tyrosine kinases (Jaks) consist of four members, Jak-1, Jak-2, Jak-3 and TYK-2. Receptors such as the IL-10 receptor, lack intrinsic kinase activity and utilise non-covalently bound Jaks for intracellular signalling. IL-10 receptor signalling has been shown to involve the stimulation of Jak1 and Tyk2 Janus tyrosine kinases (Riley et al., 1999) (see Figure 1.1.4). As shown in Figure 1.1.5, the IL-6 receptor is also associated with Jaks (Jak1 and Jak2). Ligand-mediated dimerisation of receptors associated with this pathway, results in reciprocal tyrosine phosphorylation, leading to the phosphorylation and activation of the receptor associated Jaks. Activated Jaks phosphorylate tyrosine residues on the cytoplasmic tail of the receptor, leading to the recruitment of the STAT family of transcription factors. IL-10 and IL-6, have been shown to activate STAT-1 and STAT-3 leading to the formation of homo- and hetero-dimers and in some cells STAT-5 (Finbloom and Winestock, 1995).

The utilisation of specific STAT proteins by cytokines such as IL-6 and IL-10, together with as yet unknown mechanisms of modulating the formation of homo- and hetero-STAT dimers, may in part reflect the pleiotropic effects of these cytokines and hence the flexibility of cytokine receptors to activate different genes in different situations and effect a range of contrasting biological activities within target cells. Moreover, as discussed later, signalling via the activation of STATs is interactive with other intracellular signalling pathways. Thus, STAT dimers have been shown to function as co-activators with several other classes of DNA-binding proteins, exemplified by the reported interaction of STAT-5 with the glucocorticoid receptor.
Introduction

(Stocklin., 1996). Such studies suggest that the Jak/STAT pathway may be utilised to regulate a wide range of target genes.

**IL-6 but not IL-10, Activates Transcription Factors of the C/EBP Family**

The biological functions of IL-6 are dependent on the stimulation of two distinct signalling pathways. These lead to the activation of members of the C/EBP family of transcription factors and, as discussed above, the STAT family of transcription factors (see Figure 1.1.5). NF-IL6 (nuclear factor-IL6, also termed C/EBPβ) is activated and increased synthesis of NF-IL6β (C/EBPβ) occurs following stimulation by IL-6 and both members can form heterodimers with each other, with transcriptional activity (Kinoshita et al., 1992).

**Role Of Interleukin-6 In Hsp 90 Gene Regulation**

It has previously been demonstrated that IL-6 upregulates Hsp 90 expression in the HepG2 human hepatoma cell line and in peripheral blood mononuclear cells (Stephanou et al., 1997). A role for IL-6 in Hsp 90 regulation is further supported by the observation that elevation of IL-6 in transgenic mice, results in increased levels of Hsp 90 (Stephanou et al., 1998a).

An investigation of the mechanisms of Hsp 90 promoter activation in response to IL-6, has recently been reported (Stephanou et al., 1998b). In this study, using reporter gene assays, activation of the Hsp 90β promoter occurs via the activation of the transcription factors STAT-3 and NF-IL6 and is mediated by a region of the promoter which contains binding sites for the transcription factors STAT1, STAT-3, HSF1 and NF-IL6. Interestingly, STAT-3 and NF-IL6 were observed to interact differently with HSF1 in mediating Hsp 90β gene expression.
**Introduction**

STAT-3 was observed to reduce the stimulatory effect of HSF1 while NF-IL6 enhanced it.

Further studies of the role HSF-1 and transcription factors activated by cytokines in Hsp gene expression, have demonstrated that HSF-1 and STAT-1 interact with one another via a protein-protein interaction (Stephanou et al., 1999). Moreover, as for NF-IL6, a strong synergistic effect of STAT-1 and HSF1 in activating the Hsp 90β promoter was observed. These studies would suggest an important role for transcription factors activated by cytokines, in the regulation of Hsp expression by non-stressful stimuli. Furthermore, the interaction of these non-stressful stimuli with stress responses, may reflect the apparent flexibility of the cell to activate different Hsps under different situations.

It is interesting to note that IL-6 and IL-10 utilise similar mechanisms for activating their target genes and, as discussed earlier, this is mediated via the Jak/STAT intracellular signalling pathway. Thus it is conceivable that IL-10, which activates the transcription factors STAT-1 and STAT-3, may be involved in the regulation of Hsp 90 gene expression *in vivo*.

**Summary**

The discovery of the heat shock response in *Drosophila*, has led to the identification of a multi-gene family of highly conserved proteins, which are now collectively referred to as heat shock proteins (Hsps). The regulation of Hsp gene expression by stressful stimuli has been intensively studied and further investigations into Hsp cell biology have highlighted important roles for Hsps within the cell under non-stressful conditions. However, the regulation of Hsp expression by non-stressful stimuli has been poorly understood. Recent studies have demonstrated a role for cytokines in the regulation of Hsp expression and it is likely that such non-stressful stimuli have important roles in the regulation of Hsps within the immune system. As
Introduction

will be discussed in the following section, there is now accumulating evidence that Hsp60 are involved in the development of pathogenic immune responses in a number of human autoimmune diseases, including systemic lupus erythematosus.
Section 1.2: The Role of Heat Shock Proteins in the Immune System and Pathogenesis of Autoimmune Disease

Introduction

A role for heat shock proteins (Hsps) as molecular chaperones involved in the assembly and disassembly of proteins within the cell, has been discussed in Section 1.1 of this chapter. Consistent with this role, the assembly of a diverse range of multimeric complexes produced by cells of the immune system, requires the assistance of chaperones. Thus, immunoglobulins, T-cell receptors and gene products of the major histocompatibility complex (MHC), which are all discussed later, are dependent on the function of Hsps. An important role for Hsps in protective immunity has also been suggested and is supported by the observation that Hsps of invading microbes serve as antigens in the host immune response to infection. Interestingly, a growing number of studies have also suggested that Hsp-specific immune responses, may be involved in the pathogenesis of various autoimmune diseases.

This section begins with a brief outline of the functions of the immune system, focussing on the mechanisms by which cells of the immune system specifically target foreign pathogens, while discriminating against a multitude of host-derived molecules. This is followed by a summary of various studies which suggest protective roles for Hsps in the immune system, particularly in the host immune response to microbial infection. Finally, various studies which suggest pathogenic roles for Hsps in the immune system are discussed in relation to their relevance to the development of autoimmune disease.
Introduction

Self/Non-Self Discrimination Within the Immune System

Immunology is the study of the mechanisms by which the body defends itself from invading microorganisms (including viruses and bacteria) which can cause disease, as well as the development of other disorders including cancer. Immunity is dependent on a vast array of soluble factors and cells which circulate in the blood and extracellular fluid. Three properties of this defence system are essential for virtually all immune responses: (1) a highly specific response is required for the recognition and targeting of foreign molecules; (2) the ability to discriminate foreign molecules from the multitude of host molecules is important for preventing autoreactive responses. Furthermore, (3) the acquisition of immunological memory, permits a more refined and rapid response following a repeat challenge from a specific foreign pathogen. This section focuses on the cellular and molecular basis of specificity in the immune response. However, a broad review of the immune system, can be found in Roitt, (1998).

Studies into the mechanisms which give rise to immunologic specificity and memory, have focussed on a subset of cells of the immune system which are termed lymphocytes. From these studies, several different types of lymphocytes are distinguished on the basis of their functional properties, molecular genetics and organisation. As outlined below, the ability of the immune system to respond in a specific manner to a vast number of different pathogens, is facilitated by the clonal distribution of receptors, which are expressed on the cell surface of lymphocytes.

Lymphocytes

A normal human adult body contains approximately $10^{12}$ lymphocytes, the majority of which appear to have uniform characteristics under light microscopy. However, several different types of lymphocytes may be distinguished on the basis of their functional properties and by their expression of specific proteins.

Lymphocytes may be divided into two major lineages known as B (bone-marrow-derived) cells and T (thymus-derived) cells. These account for approximately 10% and 75% respectively, of the total number of lymphocytes in peripheral blood.
**Introduction**

The remainder of the lymphocytes belong to a separate and less well characterised lineage termed natural killer (NK) cells.

B and T cells originate from stem cells in the bone marrow, which have become committed to a lymphoid (any lymphocyte lineage) line of development. A review of lymphocyte development can be found in Ikuta et al., (1992). As outlined below, the development and maturation of B and T cells from these progenitor stem cells occurs in both the bone marrow (B and T cells) and thymus (T cells only). These organs are essential for the initial production of lymphocytes (a process known as lymphopoiesis) and are collectively referred to as the primary lymphoid organs. Following dispersal into the bloodstream, these “virgin” lymphocytes migrate into various secondary (or peripheral) lymphoid organs, including the tonsils or lymph nodes. At these sites, lymphocytes frequently encounter infectious pathogens and thus it is from the secondary lymphoid organs, that immune responses are commonly initiated. A review of the role of lymphoid tissues in resistance to infection can be found in Szakal et al., (1989).

Encounters of a lymphocyte with a foreign pathogen leads to a process referred to as activation, during which successive rounds of lymphocyte cell division occur. The resulting progeny may revert to a resting state (termed memory lymphocytes) or they may differentiate into effector cells, which have a short life-span (a few days) and have important anti-microbial functions. A review of lymphocyte activation can be found in Janeway and Bottomly, (1994).

**B Cells**

The *de novo* synthesis of B cells occurs exclusively in the bone marrow and is constant throughout life. The defining feature of B cells is their unique ability to synthesise an extremely diverse family of proteins called immunoglobulins. Each immunoglobulin molecule can bind specifically to a molecular determinant of foreign or self origin. Collectively these molecular determinants are referred to as antigens. B cells express immunoglobulin in two distinct functional forms. Mature non-dividing (resting) B cells secrete large quantities of immunoglobulin onto the cell surface. The interaction of surface immunoglobulin (sIg) with other surface components
comprising disulphide-linked heterodimers of Ig-α and Ig-β, forms B cell-receptor complexes which act as specific B cell antigen receptors. Immunoglobulin expression in certain B cells (plasma cells) leads to large quantities being secreted into the blood circulation with high specificity for the antigen and these immunoglobulin molecules are referred to as antibodies.

**T Cells**

T cells develop from immature precursors in the bone marrow which migrate to the thymus which produces the hormones necessary for T cell maturation and proliferation. A review of T cell development can be found in Robey and Fowlkes, (1994). The surface expression of a number of molecules allows T cells to recognise foreign antigens. Unlike B cells, T cell recognition of an antigen requires presentation of the antigen to the T cell by specialised cells, termed antigen presenting cells (see antigen processing and presentation later in this section).

T cells may be subdivided into major classes according to the expression of specific surface molecules. The definitive marker for T cells is the T cell receptor, which is expressed on the cell surface. In humans, the majority of T cells surface express a T cell receptor (TCR) consisting of two transmembrane polypeptides, known as the α-chain and the β-chain, as well as accessory CD4 or CD8 receptors. A minor population (which is discussed later) expresses a TCR consisting of a γ-chain and a δ-chain and rarely expresses either CD4 or CD8 receptors, but functions like the αβ-TCR-expressing T cells (Kaufmann, 1996).

T cells expressing the αβ-T cell receptor, usually express only one of the CD4 and CD8 receptors and are commonly referred to as single-positive (CD4 or CD8) lymphocytes. CD8 T cells function in the killing of host cells, particularly cells expressing foreign antigens on the cell surface. This is particularly important in the identification and disposal of viral infected cells displaying viral peptides on the cell surface. CD4 T cells function to provide help for various important immunological functions of other cells, including CD8 T cells, B cells and macrophages and is reviewed in Janeway, (1999). As mentioned above, the absence of both CD4 and CD8 receptors (referred to as double-negative T cells) is observed in a small
percentage of T cells, usually of the γδ-TCR type. As discussed later, these cells appear to have important roles in the recognition of Hsp antigens of invading microbes. The individual roles of these T cell subsets and the mechanisms leading to their activation following antigen stimulation, are reviewed in Ullman et al., (1990) and Podack and Kupfer, (1991).

**Thymic Selection of T Cells and Self-Tolerance**

The ability of the immune system to distinguish foreign antigens from a multitude of host molecules, involves the elimination or functional inactivation of lymphocytes which express receptors for self-determinants and is a process fundamental to maintaining self-tolerance. Since B cell responses are largely dependent on T cell help, such selective pressures occur mainly on T cells.

As discussed earlier, progenitor T cells which develop from bone marrow, undergo maturation in the thymus. Positive selection of T cells expressing TCRs which can bind to antigens associated with MHC molecules, occurs in the thymic cortex. This process may involve interactions of T cells with epithelial cells which surface express self-peptides and one in which T cell activation provides a survival signal. In contrast, negative selection primarily occurs in the thymic medulla and leads to the deletion of T cells which express TCRs capable of recognising self-molecules. The mechanisms of thymic selection are discussed and reviewed in Janeway, (1999).
Introduction

Heat Shock Proteins and the Immune System

Antigen Processing and Presentation

The capture, processing and presentation of antigen to T cells is carried out by antigen presenting cells (APCs) and is a process fundamental to the acquired immune response. Macrophages and dendritic cells of the spleen and lymph nodes, together with B cells, are the major cell types to present antigen to T cells. APCs capture antigen by a variety of methods, including phagocytosis (principally by macrophages), pinocytosis or receptor-mediated endocytosis. The processing of foreign antigens and endogenously synthesised proteins such as viral and tumour antigens, involves their proteolytic cleavage into smaller antigenic peptides, which then associate with class I or class II MHC molecules. These peptide/MHC complexes are then transported to the cell surface where they are accessible for interaction with the T cell receptor. For a review of antigen processing and presentation see Germain and Margulies, (1993).

Endogenously synthesised proteins, including viral and tumour antigens, are degraded in the cytoplasm by a multimeric enzymatic complex (the proteosome) and translocated into the endoplasmic reticulum where they bind to class I MHC molecules. T cells expressing the CD8 receptor, which function in the lysis of their target cells, recognise peptides that are presented by MHC class I molecules on the surface of these cells. In contrast, CD4+ T cells recognise peptides (usually of extracellular origin) that are presented by class II MHC molecules on the surface of specialised APCs, including macrophages, dendritic cells and B cells.

A number of studies have revealed roles for Hsps in the folding and assembly of MHC-peptide complexes. For example, heat shock has been demonstrated to enhance the processing and presentation of antigen entering B cells (Cristau et al.,
Introduction

Studies using a mouse fibroblast cell line, have demonstrated heat shock to dramatically increase the expression of an MHC Class I-like molecule (Qa-1), which may be involved in the presentation of Hsp antigens to γδ-T cells (Imani and Soloski, 1991). Members of the Hsp 70 family have been shown to be involved in the intracellular assembly of processed-antigen/Class II MHC complexes (Vanbuskirk et al., 1989), (DeNagel and Pierce, 1992), (Schirmbeck and Reimann, 1994), (Williams and Watts, 1995) and an interaction of Hsp 70 with misfolded class II MHC molecules has been reported (Schaiff et al., 1992). Furthermore, the transport of peptides from the proteosome to the endoplasmic reticulum (ER) and their subsequent association with class I MHC molecules, is understood to involve both cytoplasmic and ER members of the Hsp 70 and Hsp 90 families (Li and Srivastava, 1993), (Srivastava, 1993), (Srivastava et al., 1994).

Taken together, these studies suggest important functions for Hsps in the folding and assembly of MHC molecules in the ER. Furthermore, Hsps appear to play a key role in the transport of antigenic peptides to the ER and the formation of stable peptide/MHC complexes, for subsequent presentation to the T cell receptor.

Hsps and Infection

The induction of Hsp synthesis in parasites during infection is likely to occur as a response to stressful conditions in the host environment and is vital for pathogen survival (Polla, 1991). Although the role of Hsps in protective host immune responses is poorly understood, numerous studies have provided evidence that Hsps of invading microbes serve as important antigenic targets in the host immune response to such infections (Kaufmann, 1990; 1991), (Kaufmann et al., 1990). Thus, both humoral and cellular immune responses to Hsps have been observed in infectious diseases involving bacteria, (Cohen and Young, 1991), fungi (Matthews and Burnie, 1992) and protozoa (Jendoubi and Bonnefoy, 1988).
A role for Hsps as important antigenic targets in the host immune response to infection, is likely to derive from several contributory factors. The induction of Hsp synthesis in microbes in response to stressful conditions such as infection, contributes to the abundance of Hsps in infecting microbes (Polla, 1991). Moreover, as discussed in Section 1.1 of this chapter, a high degree of sequence homology is observed for Hsp family members (particularly Hsp 90, Hsp 70 and Hsp 60), between microbial species. For example, the Mycobacterium Hsp 60 and GroEl of _E.coli_, share a broad range of immunological cross-reactive determinants (Shinnick et al., 1988). Thus, infection and exposure of the immune system to microbial Hsps leads to immunologic memory which following subsequent infections, is capable of recognising and responding to Hsps from a range of different organisms.

These factors therefore, are likely to explain the prominence of Hsps as major antigenic determinants of infecting microbes and the importance of host anti-microbial Hsp responses in promoting the efficient recognition and clearance of such infections.

Cell Surface Expression of Heat Shock Proteins

As discussed earlier in this section, Hsp determinants are expressed on the cell surface following microbial infection. Indeed, their subsequent presentation in the context of different MHC molecules and the generation of Hsp-specific immune responses, has been suggested to be important to the host immune response to microbial infection. The mechanisms responsible for the relocalisation of Hsps (which are typically cytoplasmic) to the cell surface, are at present poorly understood.

Various studies have demonstrated such relocalisation in different situations. For example, Hsp 90 (La Thangue and Latchman, 1988), (Ferrarini et al., 1992), Hsp 70 (Poccia et al., 1992) and Hsp 60 (Fisch et al., 1990), (Kaur et al., 1993), have been observed to be surface expressed by lymphoma cells. Hsp surface expression has also
been reported for non-transformed cells. For example in patients with SLE, Hsp 90 is surface expressed by peripheral blood lymphocytes (Erkeller-Yuksel et al., 1992). As shown in Table 1.2.1 and discussed later, studies of other autoimmune diseases have also reported the surface expression of Hsps, particularly at sites of active tissue disease.

**Heat Shock Proteins and Autoimmune Disease**

The process of self-tolerance is therefore fundamental to the normal function of the immune system and requires the recognition of a potentially infinite variety of foreign antigens, while discriminating against self antigens. However, as will be discussed, a loss of self-tolerance involving the activation of self-reactive lymphocytes, are defining features of autoimmunity, with possible pathological consequences. Importantly, as shown in Table 1.2.1, studies of various autoimmune diseases have reported an increased prevalence of self-Hsp-specific responses in patients, involving both antibodies and T cells. Moreover, in some cases these responses have correlated with active disease.

**Antibodies to Heat Shock Proteins**

Antibodies to Hsps have been found in a number of human autoimmune diseases. In SLE of both adult (Conroy et al., 1994) and childhood onset (Conroy et al., 1996), raised serum levels of antibodies to Hsp 90 have been reported. Overexpression of Hsp 90 in PBMCs has been observed in a subset of patients with SLE and in these patients this correlated with active central nervous system and cardiovascular/respiratory disease (Dhillon et al., 1994). Interestingly, increased surface expression of Hsp 90 in PBMCs and raised antibodies to Hsp 90, correlated with a subset of patients which has been associated with Hsp 90 overexpression (Conroy et al., 1994).
Table 1.2.1  Immune Response to Hsps in Human Autoimmune Diseases

<table>
<thead>
<tr>
<th>Autoimmune Disease</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic lupus erythematosus</td>
<td>Overexpression of Hsp 90 in PBMCs (Dhillon et al., 1994). Raised antibody levels to Hsp 90 in patients of adult onset (Conroy et al., 1994) and childhood onset (Conroy et al., 1996).</td>
</tr>
<tr>
<td>Sjogren's syndrome</td>
<td>Gastric involvement and Helicobacter pylori previously reported. Increased prevalence of antibodies against Hsp 60 of Helicobacter pylori.</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>Raised levels of antibodies to mycobacterial Hsp 60 (Tsoulfa et al., 1989) which react with synovial tissue (de Graeff-Meeder et al., 1990). Increased frequency of B cells specific for mycobacterial Hsp 60 in synovial fluid (Rudolphi et al., 1997).</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Increased prevalence of antibodies to alpha-crystallin correlate with active disease and disease severity (Agius et al., 1999).</td>
</tr>
<tr>
<td>Atherosclerosis</td>
<td>Levels of antibodies against mycobacterial and human Hsp 60/65 are increased in patients with atherosclerotic lesions (Kleindienst et al., 1995), (Schett et al., 1997), (Metzler et al., 1998).</td>
</tr>
<tr>
<td>Coeliac disease</td>
<td>Enhanced expression of epithelial cell mitochondrial Hsp 65 in patients with this disease correlates with significantly higher gammadelta+ T cell densities than those with normal Hsp 65 expression (Iltaenen et al., 1999).</td>
</tr>
</tbody>
</table>
**Introduction**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Behcet’s disease</td>
<td>T cells in this disease proliferate vigorously in an antigen-dependent manner in response to a specific peptide of human Hsp 60 (Sakane, 1997), (Kaneko et al., 1997).</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>Antibodies to mycobacterial Hsp 60 in serum of patients (Rambukkana et al., 1993), (Izaki et al., 1994)</td>
</tr>
<tr>
<td>Autoimmune thyroiditis</td>
<td>Increased prevalence of IgM antibodies to human Hsp 72 and Hsp 73 in serum of patients (Appetecchia et al., 1997).</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td>Patients have increased prevalence of antibodies to Hsp 72, without correlation with any disease characteristic (Prummel et al., 1997).</td>
</tr>
<tr>
<td>Menier’s disease</td>
<td>Increased prevalence of antibodies to Hsp 70 in serum of patients (Shin et al., 1997).</td>
</tr>
</tbody>
</table>
**Introduction**

Increased levels of antibodies to Hsp 70 have also been reported in various autoimmune diseases, such as Menier's disease (Shin et al., 1997). However, any involvement of Hsp 70 in autoimmune pathogenesis remains unclear. For example, studies of patients with autoimmune thyroiditis (Appetecchia et al., 1997) and grave's disease (Prummel et al., 1997) showed raised antibody levels to Hsp 70, without correlation with disease activity. In multiple sclerosis (MS), increased expression of Hsps occurs in various cells of the central nervous system during active disease (Birnbaum, 1995), (Aquino et al., 1997) and Hsp 70-specific antibodies have been detected in serum and cerebrospinal fluid of patients. A role for antibodies to Hsp 70 in autoimmune pathology has been suggested and is supported by the observation that antibodies to Hsp 70 react with human myelin proteins of normal individuals and with oligodendrocytes in regions of MS demyelination (Birnbaum, 1995). Thus it is possible that immune responses to Hsps or cross-reactive Hsp epitopes, may influence disease progression, possibly involving demyelinating processes in patients with MS (Birnbaum and Kotilinek, 1999).

In RA, a number of studies have reported humoral responses to Hsp 60 and in some cases, these responses have been linked to affected tissues. For example, raised levels of antibodies to mycobacterial Hsp 60 have been reported in RA (Tsoulfa et al., 1989), (McLean et al., 1990) and have been reported to be reactive with synovial tissue in patients with RA (de Graeff-Meeder et al., 1990). Furthermore, an increased frequency of B cells specific for mycobacterial Hsp 60 has been found in the synovial fluid and tissues of affected joints, in patients with RA (Rudolphi et al., 1997). Moreover, in another study, intra-articular administration of mycobacterial Hsp 65 in Wister rats sensitised to M. tuberculosis, induced the production of antibodies to Hsp 65 and joint inflammation, supporting the hypothesis that Hsp 65 is an arthritogenic stimulus (Winrow et al., 1994). In juvenile chronic arthritis (JCA), raised levels of antibodies to human Hsp 60 have been reported, particularly in those patients with polyarticular onset (de Graeff-Meeder et al., 1993). Infection with the bacterium *Helicobacter pylori* may be linked to gastric involvement in patients with Sjogren's disease and raised levels of antibodies to Hsp 60 of *H.pylori* have been reported in this disease (Aragona et al., 1999). Increased levels of antibodies specific to mycobacterial Hsp 60 have also been reported in patients with psoriasis (Izaki et al.,
1994) and increased levels of these antibodies have been observed to correlate with active disease (Rambukkana et al., 1993). In atherosclerosis, (Kleindienst et al., 1995), (Schett et al., 1997), levels of antibodies to mycobacterial and human Hsp 60 are increased in patients with atherosclerotic lesions. Moreover, macrophages in atherosclerotic lesions have been shown to express high levels of Hsp 60 in these patients.

T Cell Responses to Heat Shock Proteins

T cell responses to Hsps have been reported in a number of autoimmune diseases. As for the antibody responses detailed above, it is often found that the prevalence of these responses is higher in patients than controls. Moreover, as will be discussed, overexpression of self-Hsps in regions of tissue inflammation have been linked to these responses and shown to correlate with disease activity.

In multiple sclerosis (MS), αβ-T cells reactive with Hsp 70 and Hsp 60 are more prevalent in patients with this disease compared to controls (Salvetti et al., 1992), (Birbaum et al., 1993). Moreover, the response of αβ-T cells to Hsp 70, involved the cross-recognition of both microbial Hsp 70 and self Hsp 70, implicating Hsp 70 as a potential autoantigen in MS (Salvetti et al., 1996). In rheumatoid arthritis (RA), overexpression and cell-surface localisation of Hsp 60, has been reported on synovial lymphocytes (Sato et al., 1996) and αβ-T cells from inflamed sites have been demonstrated to be reactive to both mycobacterial and self-Hsp 60 (Li et al., 1992). In another study of the reactivity of both synovial and peripheral blood T cells to Hsp 60, T cells isolated from patients with RA and normal controls, were found to be preferentially reactive to mycobacterial Hsp 60 and not human Hsp 60. Evidence of in vivo activation and clonal expansion of these mycobacterial Hsp 60-reactive T cells was also reported (Celis et al., 1997). In Behcet’s disease, T cells isolated from patients have been observed to proliferate vigorously to a specific peptides of both human (Sakane, 1997) and mycobacterial (Pervin et al., 1993) Hsp 60.
Introduction

Summary

Studies supporting a functional role for Hsps in the immune system, particularly in antigen processing and presentation, have been discussed in this section. Moreover, accumulating evidence has been summarised, supporting a role for Hsps as immunodominant antigens in protective host immune responses to microbial infection. This observation may in part be explained by high sequence homology between Hsps from different microbes.

A role for Hsps in pathogenic immune responses in various human autoimmune has also been outlined. However, an understanding of the mechanisms responsible for such responses and their contribution to the development of autoimmune disease, is unclear. Interestingly, it appears likely that in certain situations, such as autoimmune disease, sequence homology between Hsps may contribute to the development of cross-reactive pathogenic T cell responses (a process known as "molecular mimicry"). As discussed earlier, this situation may occur in various autoimmune diseases and involve the elevated expression and surface localisation, of Hsps at sites of tissue inflammation. Thus, it is likely that such a role for Hsps, rather than initiating a pathogenic autoimmune response, may enhance and occur secondary to the presence of active tissue disease. As has been discussed, a number of contributory factors are involved in the development of autoimmune disease. The role of Hsps in SLE is discussed in detail in Section 1.4 after a discussion of SLE itself in Section 1.3.
Introduction

Section 1.3: Systemic Lupus Erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a human autoimmune rheumatic disease and descriptions of its various manifestations have been described in literature dating back many centuries. Research towards an understanding of the mechanisms in SLE which leads to widespread tissue damage, involving many of the body’s organs or systems, has been aided greatly by the development of several animal models. From all these studies, it is certain that SLE develops as a result of the complex interaction of a variety of factors: genetic, hormonal and environmental. At present however, the specific aetiology of this disease remains unclear, and is the subject of much debate. In this section, the variety of clinical manifestations which characterise SLE are summarised. This is followed by an outline of the various immunological abnormalities which are observed in patients with SLE and their potential pathological consequences.

Definition And Classification

SLE may be defined as a disease with a complex multifactorial aetiology, characterised by inflammation and the involvement of most of the body’s organs and systems. Not surprisingly, the expression of this disease covers a wide clinical spectrum and may overlap with other autoimmune rheumatic diseases such as rheumatoid arthritis, dermatomyositis/polymyositis and Sjogren’s disease. Such diversity in clinical presentation with no uniquely identifying characteristic (Snaith, 1992), has necessitated a consensus of agreement on criteria for the classification of SLE. In 1971, the American Rheumatism Association (ARA), now the American College of Rheumatology (ACR), published criteria for the classification of SLE and these are discussed in Cohen and Canoso, (1972). These criteria have been revised by the Diagnostic and Therapeutic Committee of the ACR (Tan et al., 1982), (Hochberg,
Introduction

1997) and currently comprise 11 manifestations which may be observed in SLE. Of these (see Table 1.3.1), the ACR has proposed that a person with SLE must present with a minimum of four, serially or simultaneously, during any period of observation. These criteria have been tested for their sensitivity for SLE and specificity against other autoimmune diseases. Using an ACR database of 590 patients with SLE, scleroderma or dermatomyositis/polymyositis, the ACR criteria published in 1982 (Tan et al., 1982), were found to be 83% specific for SLE and 89% specific against the combined scleroderma and dermatomyositis/polymyositis patients (Fries, 1987). Although the ACR criteria were intended to be used as a classification tool appropriate for use in clinical trials, its use has combined with the need for a practical case definition of a patient with SLE. Problems however, with the use of the ACR criteria for diagnostic purposes have been highlighted (Levin et al., 1982). In this report, less than 50% of patients studied, fulfilled the ACR criteria at any one time. Furthermore, in some patients, fulfilling the criteria required a period of observation of between 9 and 20 years.
Table 1.3.1  Criteria of the American College of Rheumatology for the Classification of SLE from Tan et al., (1982), Hochberg, (1997)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
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<tbody>
<tr>
<td>1) Malar Rash</td>
<td>Fixed malar erythema, flat or raised.</td>
</tr>
<tr>
<td>2) Discoid Rash</td>
<td>Erythematous-raised patches with adherent keratotic scaling and follicular plugging. Atrophic scarring may occur in older lesions.</td>
</tr>
<tr>
<td>3) Photosensitivity</td>
<td>Skin rash occurring as an unusual reaction to sunlight.</td>
</tr>
<tr>
<td>4) Oral Ulcers</td>
<td>Oral or nasopharyngeal ulcers.</td>
</tr>
<tr>
<td>5) Arthritis</td>
<td>Non-erosive, characterised by tenderness, swelling or effusion. Involving two or more peripheral joints.</td>
</tr>
<tr>
<td>6) Serositis</td>
<td>(a) Pleuritis or (b) Pericarditis.</td>
</tr>
<tr>
<td>7) Renal Disorder</td>
<td>(a) Persistent proteinuria ( &gt; 0.5g/24 hr or 3+) or (b) Cellular casts.</td>
</tr>
<tr>
<td>8) Neurologic Disorder</td>
<td>(a) Seizures or (b) Psychosis (in absence of other causes).</td>
</tr>
<tr>
<td>9) Hematologic Disorder</td>
<td>(a) Haemolytic anaemia or (b) Leukopenia (&gt; 4.0 x 10^9/ml on two or more occasions) or (c) Lymphopenia (&gt; 1500/ml on two or more occasions) or (d) Thrombocytopenia (&lt; 1 x 10^5/ml).</td>
</tr>
<tr>
<td>10) Immunologic Disorder</td>
<td>(a) Raised anti-double-stranded DNA antibody binding or (b) Anti-Sm antibody or (c) Positive finding of antiphospholipid antibodies based on: (1) an abnormal serum level of IgG or IgM anticardiolipin antibodies, (2) a positive test result for lupus anticoagulant using a standard method, or (3) a false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by <em>Treponema pallidum</em> immobilization or fluorescent treponemal antibody absorption test.</td>
</tr>
<tr>
<td>11) Anti-nuclear Antibody</td>
<td>Raised titre of anti-nuclear antibody.</td>
</tr>
</tbody>
</table>
Epidemiology

The widespread use of the ACR criteria for the classification of SLE, has facilitated the epidemiological study of this disease. Thus, information obtained from patients with SLE world-wide, has allowed good estimates to be made for prevalence, morbidity and prognosis/survival in this disease. In addition, such population studies are valuable for both the development and subsequently clinical trials, of potential therapeutic drugs in SLE.

Prevalence

SLE is recognised as a disease with a world-wide distribution and primarily affects females of child-bearing years (Morrow et al., 1999). Studies to estimate the prevalence of SLE in various countries, have highlighted geographical differences in the reported figures. In Japan (Nakae et al., 1987) and Sweden (Nived et al., 1985), a prevalence of 21 and 39 cases of SLE respectively per 100,000 of total population, have been reported. In the United States, estimates of the prevalence of SLE have been reported to range between 14.6 (Siegel and Lee, 1973) and 50.8 cases (Fessel, 1974), per 100,000 persons and a later study in 1985 (Michet et al., 1985), reported an overall figure of 40 cases per 100,000 persons. More recently however, Hochberg et al., (1995) used a method of telephone-screening for physician diagnosed SLE in women aged 18 years and older and suggested previous studies to under-estimate the prevalence of SLE in the USA by at least 3-fold. In Europe, prevalence figures have ranged from 12.5 per 100,000 females in England (Hochberg, 1987) to a high of 39 per 100,000 people (both sexes combined) in Sweden (Jonsson et al., 1989).

Such variability in estimates of prevalence are likely in part to reflect differences in the methodology used in each study, for case ascertainment. More significantly, an ethnic bias for the prevalence of SLE is recognised. Two studies in the United Kingdom, have highlighted significant variation in the prevalence of SLE among different ethnic groups sharing similar urban environments. In Nottingham, UK, the prevalence of SLE was reported as 3.7 and 45.4, per 100,000 males and females respectively. The authors also reported that SLE was found to be more
Introduction

prevalent amongst Afro-Carribean groups (Hopkinson et al., 1993). A similar study in Birmingham, UK, reported significant differences in the prevalence of SLE depending on ethnic group and irrespective of place of birth. Prevalence rates of 36.2, 90.6 and 206 per 100,000 women were reported for Caucasian, Asian and Afro-Carribean ethnic groups respectively (Johnson et al., 1995).

Reports however, of the relevance of ethnicity in population studies to the prevalence of SLE, can be conflicting. For example, in the United States and Caribbean, approximately 1 in 500 black women suffer from SLE, however it appears that SLE is uncommon among African blacks (Fessel, 1988). Thus, all these studies ultimately suggest a complex multi-factorial aetiology in SLE and it is likely that a number of genetic (discussed later), environmental and sociological factors combine to cause this disease.

Clinical Features of SLE

A wide spectrum of clinical features are observed in patients with SLE, as varied as skin rashes and kidney failure. The treatment of any one clinical feature may be followed by the later emergence of others, with unpredictable severity. Thus, periods of mild illness, which often involve the skin and joints (Worrall et al., 1990), may be followed by major involvement of the kidney, lung or central nervous system. Although SLE may once have been thought of as fatal, the wide-spread use of tests for measuring the titres of autoantibodies associated with SLE pathogenesis (notably anti-nuclear antibodies), has facilitated the identification of mild forms of the disease. More judicious use of immunosuppressive drugs and the development of dialysis and renal and heart/lung transplant, have all combined to improve the survival figures from 50% at five years in 1950, to 95% at five years now (see Morrow et al., 1999).
Introduction

Non-Specific Features

A number of general and non-specific features are observed in patients with SLE and do not form part of the ACR criteria. Of these, lethargy is frequently associated with active SLE (Wysenbeek et al., 1993). Swelling of the lymph glands around the neck and in the armpits may also be observed. If profound, a tissue biopsy may be required to exclude malignancy. Patients may also experience weight loss, fever, alopecia and on occasion nausea.

Dermatological Involvement

Skin manifestations are a presenting feature in many patients with SLE and these have often been noted to occur before systemic spread of the disease (McGehee et al., 1954). Cutaneous involvement can take many forms, including malar erythema (butterfly rash), nail base erythema, mucous membrane lesions, bullous lesions, maculopapular and discoid lesions, moderate or severe livedo reticularis and splinter haemorrhages. The classic butterfly rash is an erythematous and papular facial lesion involving the cheeks and bridge of the nose and may be present in up to 68% of patients (Grigor et al., 1978). Many patients are photosensitive (commonest in white females) and exposure to UV radiation may precipitate such skin problems (Nived et al., 1993). Vasculitis affecting the fingers and toes often occurs. Patchy, diffuse scarring or non-scarring alopecia is a common feature of SLE and was listed as a criteria in the initial 1971 ARA report for the classification of SLE (Cohen and Canoso, 1972). However its non-specificity to SLE and difficulties of interpretation have caused this manifestation to be excluded subsequently.

Musculoskeletal Involvement

Arthralgia in SLE is typically polyarticular, symmetrical and flitting and occurs in approximately 90% of patients with SLE, of whom half will suffer an accompanying early morning stiffness. Joint involvement tends to be mild, however approximately 5% of these patients will suffer severe arthritis with joint deformity in
Introduction

the hands (Jaccoud's arthritis) (Szczepanski et al., 1992). In such cases, examination of the synovial fluid usually demonstrates a low white blood cell count (< 3000 cells/ml) and a predominance of mononuclear cells (Pekin, 1970). Immune complex deposition has been described in synovial tissue (possibly involved in observed synovial lesions) and is often found in muscles of patients with SLE, although this may not parallel significant muscle disease (Russell and Hanna, 1988).

Renal Disease

Active renal disease is associated with morbidity in SLE. Clinically this may present with weight gain, shortness of breath, ankle swelling, haematuria and/or proteinuria. Monitoring of blood pressure and various laboratory indicators such as red or white cell casts in the urine, serum urea, is important (Esdaile et al., 1989). Renal biopsy can be used to identify immune complexes (associated with nephritis pathology) in the glomerulus, which appears to be a focal point for renal disease (McCluskey, 1982). Information from a range of histological studies relating to renal biopsy, has led to the World Health Organisation (W.H.O) to describe five categories of renal disease, ranging from normal to diffuse membranous glomerulonephritis on light/electron microscopy. Subsequently, a more detailed classification of lupus nephritis has been suggested following an international study of kidney disease in children (Churg and Sobin, 1982). The major manifestations range from minimal to advanced sclerosing glomerulonephritis, on light microscopy. The significance of renal biopsy to general prognosis in patients with SLE is debatable. It has been suggested that biopsy-derived information may facilitate predictions of renal involvement, but is less relevant to the clinical stage of the disease as a whole (Schwartz et al., 1993).
Introduction

Involvement of the Nervous System

The involvement of the nervous system in SLE has been well documented and features range from mild migraine headaches in up to 40% of patients, epilepsy as a presenting feature in 5% of cases and major psychosis. Grand mal seizures are the most common severe form of epilepsy and eventually manifest in up to 20% of patients with SLE (Morrow et al., 1999). A range of autoantibodies, including those with specificity for small nuclear ribonucleoproteins (RNPs) such as Sm RNPs (involved in RNA splicing) as well as intracytoplasmic molecules such as ribosomal-P, have been linked to neurological disease, and this is currently an area of active research (Hay and Isenberg, 1993). Nervous system involvement may be sub-divided into central/cerebral, peripheral lesions and psychological manifestations.

Central/cerebral involvement may be due to a variety of mechanisms including vasculitis, cross-reacting anti-neuronal/lymphocyte antibodies and anti-phospholipid antibodies. In addition, cerebral disease may occur as a secondary event due to biochemical disturbances such as uraemia. Immune complex deposition may contribute to diffuse manifestations and the presence of anti-phospholipid antibodies is also associated with various movement disorders in SLE, such as chorea. Psychiatric disturbances have been frequently reported in patients with SLE. These include depression and anxiety and may also be associated with an impairment of cognitive function (Rogers, 1983). These problems have been reported in up to 50% of patients with SLE (Hall et al., 1981).

Haemopoietic Involvement

Chronic normocytic anaemia (normochromic) is frequent in patients with SLE. Levels of ferritin in these patients are often normal, however iron deficiency may occur as a result of corticosteroid or non-steroidal anti-inflammatory drug therapy, which can lead to gastric bleeding. Haemolytic anaemia (Coombs’ positive) occurs in approximately 10% of patients, although this may not always be associated with haemolysis (Morrow et al., 1999).
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Leucopenia (< $4 \times 10^9$/L) and lymphopenia (< $1.5 \times 10^9$/L) are frequently observed abnormalities of white blood cell count in SLE. Such immune disturbances may be mediated by autoantibodies and lymphocytotoxic antibodies feature in approximately one third of patients. Other factors, including decreased marrow production of white blood cells and immunosuppressive consequences of corticosteroid therapy, may also contribute to the observed decrease in white blood cell count in patients with SLE.

Thrombocytopenia may be chronic (< $100 \times 10^9$/L) and is not usually associated with bleeding episodes. Acute thrombocytopenia is less frequently observed in SLE and is associated with coagulation abnormalities due to a fall in platelet count which may lead to severe or fatal bleeding episodes. These patients typically feature circulating anticardiolipin antibodies, recurrent cerebro/cardiovascular thromboses and pulmonary hypertension (Asherson et al., 1983). Such disease manifestations associated with the presence of serum anticardiolipin antibodies in patients with SLE, are collectively referred to as antiphospholipid syndrome (Cabral et al., 1992).

Cardiorespiratory/Vascular Involvement

Pulmonary disease is common in SLE and typically causes pleuritic chest pain and cough in approximately half of all patients at some time. Poor diaphragmatic movement and mild dyspnoea may be indicators of abnormal pulmonary function, particularly reduced total lung capacity and flow rates, in upto 50% of patients (Morrow et al., 1999). Diaphragmatic dysfunction in SLE may contribute to the recognised but uncommon condition “shrinking lung syndrome” (Hoffbrand et al., 1992) involving reduced chest expansion and pathologic lesions of the alveoli. Parenchymal changes in the lung associated with SLE, have been reported in 18% of patients (Haupt et al., 1981) and include interstitial fibrosis, pulmonary vasculitis and interstitial pneumonitis. A chronic non-productive cough may be present in such patients and usually accompanies shortness of breath. Following an episode of acute lupus pneumonitis, a more acute presentation may occur (Weinrib et al., 1990). Inflammatory lesions may be present in the pleural and pericardial membrane and
Introduction

may in a few patients correlate with immune complex deposition.

Management

Although there is no cure for SLE, the majority of the clinical manifestations observed in this disease can be successfully controlled. Thus, continuous monitoring of disease activity through clinical and laboratory parameters, is vitally important. Certain precautionary measures are recommended and these include reduced exposure to ultra-violet light and avoidance of stress where feasible. Joint pain, skin rashes and fatigue, can often be controlled by combinations of non-steroidal anti-inflammatory drugs such as hydroxychloroquine. The majority of immunomodulatory treatments involve corticosteroids, such as prednisolone, particularly in the presence of renal, major central nervous system and cardiovascular/respiratory involvement. In combination with steroids, other drugs such as azathioprine and cyclophosphamide, may be used for their immunosuppressive effects. However, certain side effects have been observed as a result of the over-use of these drugs. These include infection due to the immunosuppressive effects and steroids also cause osteoporosis, high blood pressure and diabetes mellitus.

Prognosis and Survival

Although SLE may once have been considered to be both a serious and fatal disease, the wide-spread use of immunosuppressive drugs has improved both prognosis and survival in patients with this disease. Most fatalities may be attributable to active disease, particularly renal, central nervous system disease and atherosclerotic vascular disease (Ginzler et al., 1982). Renal disease, as measured by serum creatinine or urine protein assessment, is the most important predictor of poor outcome (Ginzler et al., 1982), (Reveille et al., 1990). Patients receiving high-dose steroid and cytotoxic therapy may also succumb to opportunistic infections due to the immunosuppressive effects of these drugs. A long-term study of 100 patients with
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SLE in the UK, showed a 5 year survival rate of 88% and a ten year survival rate of 86% (Worrall et al., 1990). During the study period there were 13 deaths (mean age 36.5 years) and half of these were attributable to SLE (including renal and central nervous system disease, thrombocytopenic purpura). An analysis of demographic factors associated with morbidity in SLE has highlighted poorer survival rates in Indian Asians in the UK, compared to their Caucasian counterparts, with an increased frequency of renal and central nervous system complications (Samanta et al., 1991). Similar observations are found for Afro-Carribeans in the United States (Ginzler et al., 1982), (Nossent, 1993) compared to Caucasians, with a greater frequency of central nervous system involvement and higher mean serum creatinine levels. In general, non-Caucasian and poor socio-economic environment are factors which have been associated with decreased survival in a number of SLE population studies (Studenski et al., 1987).

Immunopathology In SLE

In this section, the factors which may predispose an individual to developing autoimmunity are considered. Finally, the various abnormalities observed in the immune system of patients with SLE are highlighted, and their role in the disease process discussed.

Genetic Susceptibility

The understanding that an individual’s genetic background may influence susceptibility to acquiring SLE and be involved in the disease process derives from observations in different populations; family studies and individual genetic analysis. SLE primarily affects females of child-bearing age and as already discussed (see p. 89) displays an ethnic bias towards Afro-Carribeans and to a lesser extent Orientals. Furthermore there is a greater concordance for this disease in monozygotic twins (25%) compared to 3% in dizygotic (Deapen et al., 1992). In relatives of patients with SLE there is a 3% risk of SLE and an increased prevalence of autoantibodies in
the serum of healthy relatives with SLE, reviewed in Miles and Isenberg, (1993).

Genome-wide linkage analyses have been used to identify regions of chromosomes or individual genes, in genetic linkage with disease. In humans, regions of chromosome 1 (1q21-23, 1q41-4) (Moser et al., 1998) and of the major histocompatibility complex (discussed later) region on chromosome 6 (6p11-21) (Gaffney et al., 1998), have been identified as potential lupus susceptibility loci. However, these studies also highlight the complexity of lupus genetics which differs among ethnic groups. Analysis of the region 1q23 revealed loci in genetic-linkage with SLE in African-American pedigrees, but not in European Americans (Moser et al., 1998).

Linkage analyses of murine models of SLE with different phenotypes and genotypes, have greatly aided the genetic dissection of SLE-susceptibility. The majority of these studies have been performed in New Zealand hybrid models (described later in this section) and have identified the genomic intervals Sle1, Sle2 and Sle3 respectively on chromosomes 1, 4 and 7 (Kono et al., 1994; Morel et al., 1994) as conferring strong susceptibility to lupus. Comparisons of potential human susceptibility loci in SLE with studies in murine models of this disease show similarities. For example, the Sle1 susceptibility region on mouse chromosome 1 in New Zealand models is syntenic with the human SLE susceptibility interval 1q23-31, which contains the locus for the Fc receptor gene FcyR2, shown to be in disease-linkage in both African- and European-Americans (Moser et al., 1998) and Korean populations (Song et al., 1998). Hence, genetic synteny between human and murine susceptibility regions suggests shared susceptibility genes and thus highlights the potential of murine models of SLE and congenic mouse strains to aid our understanding of lupus genetics in humans. Various candidate human SLE susceptibility genes are now discussed.

**Sex Hormones**

Female susceptibility to SLE over males implies the involvement of sex hormones. Experimental and human models of SLE have shown that sex hormones affect the immunological features of the disease. Androgens such as testosterone tend
Introduction

to elicit an immunosuppressive effect, while oestrogens are immunoenhancing through B cell stimulation and regulatory suppression. (Ansar Ahmed et al., 1985). Treatment with testosterone in MRL-lpr/lpr mice (a lupus-prone mouse model), is observed to reduce lupus-like symptoms without affecting lymphoproliferation (Carlsten et al., 1989).

Major Histocompatibility Complex

Research into the involvement of genes of the major histocompatibility complex (MHC) in human SLE has identified haplotypes associated with disease susceptibility and to autoantibody subsets (particularly MHC Class II genes). It is however important to note that any correlations between MHC haplotype and SLE must be understood in relation to ethnic background.

In caucasians, the composite haplotype HLA A1, B8, DR3 is present in 35% of caucasians with SLE, compared with 6% in caucasian normals. The presence of both A1 and B8 alleles was associated with a relative risk of 8.0 and both B8 and DR3 alleles with a relative risk of 8.32 (Worrall et al., 1990). In early-onset SLE there is an association with the DRw8 haplotype, while the frequency of neuropsychiatric involvement negatively correlates with DQ. Antibodies to DNA, nuclear and cytoplasmic antigens in patients also correlate with MHC (Arnett., 1997). HLA DR2 is associated with antibodies to Sm in Afro-Carribbeans and these individuals show a reduced frequency of DR3. DR4 is associated with autoantibody production to RNP and confers a reduced risk to nephritis, while a reduced susceptibility to nephritis correlates with the DR2 haplotype.

Non-HLA genes within the MHC have also been suggested to be involved in the pathogenesis of this disease. For example, polymorphisms within the tumour necrosis factor gene are associated with an increased susceptibility to SLE (Tarassi et al., 1998; Rood et al., 2000) while the genes encoding the complement components C2 and C4 (discussed below) have also been associated with SLE. Of particular interest to this dissertation is the regulation of the Hsp 90β gene in SLE (see Section 1.4 of this chapter) and the reported localisation of this gene on human Chromosome 6, in the MHC region (Durkin et al., 1993; Takahashi et al., 1994). The reported
differential regulation of this gene in genetic subsets of patients with SLE (Dhillon et al., 1994), implicates the existence of Hsp 90β promoter polymorphisms encoded onto particular HLA haplotypes. However, no studies have yet established the presence of such polymorphisms. Table 1.3.2 shows the chromosomal location of the genes of the major Hsp families in humans and mice.

**Complement Deficiencies**

Complement deficiency refers to a decrease in function of the complement system and is a feature of a number of autoimmune diseases including SLE. Activation of the complement system through the formation of immune complexes is intimately linked with autoimmune pathology. Deposition of immune complexes on tissue surfaces can result in a focus of inflammation which if perpetuated, may lead to tissue destruction. Complement deficiency in autoimmune disease can in part reflect commitment of the complement system to immune complex clearance.

The recognition of hereditary and inborn abnormalities of complement, and their correlation with clinical features of autoimmune disease, has prompted an understanding of complement genetics (for a review see Walport et al., 1997). The loci for some of the genes encoding complement components, notably C2 and C4, are found within the HLA region and are referred to as HLA class III antigens. HLA haplotype associations with complement deficiency are recognised for such components.

SLE is associated with deficiencies of the early classical pathway components, most strongly with the complement components C1q, C1r, C1s, C2 and C4. Each component is inherited through two alleles and deficiency of either may therefore be partial (heterozygous deficiency), or complete (homozygous deficiency) (see Kemp et al., 1987). Congenital deficiencies of C2 and C4 are frequently in linkage disequilibrium with HLA DR2 and DR3, (see Schur, 1995). C4 comprises two distinct forms, C4A and C4B. A large number of alleles have been identified which may occupy each locus, including null alleles (Mauff et al., 1990). A C4A null allele is a common feature of complement deficiency and a single null allele increases the risk for developing SLE by 3 and two null alleles by 17. C4A deficiency is
### Table 1.3.2 Location of Hsp Genes in Disease Susceptibility Intervals in Human and Mouse Chromosomes

**Humans**

<table>
<thead>
<tr>
<th>Hsp</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp 90</td>
<td>1p22.1</td>
</tr>
<tr>
<td>Hsp 60</td>
<td>3p22.3</td>
</tr>
<tr>
<td>Hsp 90β</td>
<td>6p21.1 – HLA</td>
</tr>
<tr>
<td>Hsp 90α</td>
<td>14p32.21 – not linked</td>
</tr>
</tbody>
</table>

**Mouse**

<table>
<thead>
<tr>
<th>Hsp</th>
<th>Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp 40</td>
<td>C5</td>
</tr>
<tr>
<td>Hsp 40</td>
<td>C4</td>
</tr>
<tr>
<td>Hsp 105/Hsp 25</td>
<td>C5</td>
</tr>
<tr>
<td>Hsp 70-1</td>
<td>C17</td>
</tr>
<tr>
<td>Hsp 84-1</td>
<td>C17</td>
</tr>
<tr>
<td>Hsp 70-2/Hsp 86-1</td>
<td>C12</td>
</tr>
<tr>
<td>Hspa4</td>
<td>C11</td>
</tr>
<tr>
<td>Hspg2 (perlecam)</td>
<td>C4</td>
</tr>
</tbody>
</table>
associated with impairment of immune complex clearance and solubilisation, leading to deposition in lungs and kidneys and subsequent inflammation. C4 deficiency may occur through a mutation within the C4 coding region, resulting in a deletion of C4A and part of C4B and is associated with DR3+ patients (Kemp et al., 1987). In drug-induced SLE, inhibition of C4A and C4B activity can occur due to binding of the drug to C4 active sites, thereby interfering with binding to immune complexes.

The expression of complement receptors CR1 and CR2 in phagocytes and T cells is significantly reduced in SLE (Wilson et al., 1986). Low CR1 expression on erythrocytes (Walport et al., 1985) and peripheral blood leucocytes (Wilson et al., 1986) has been reported and is seen to correlate with disease activity. CR2 receptor expression on CD4+ and CD8+ peripheral T cells is increased in some patients with active SLE and may lead to increased T cell cytotoxicity. B cells in patients with active and inactive SLE show reduced CR2 expression (Wilson et al., 1986) and may be associated with abnormal cytokine regulation.

Immune Dysregulation and Cellular Abnormalities in SLE

Immune disturbances involving lymphocyte and accessory cell abnormalities together with cytokine dysregulation are characteristic features of SLE immunopathology (for a review see Kirou and Crow, 1999). Circulating B and T lymphocyte numbers may be increased or decreased and may vary with disease activity. B lymphocyte hyperactivity and failure of T lymphocytes to suppress autoreactive B cell clones, leads to the formation of antibodies (40 -50 types) some of which are likely to be pathogenic, see Linker-Israeli et al., (1990). Cytokine dysregulation plays a pivotal role in the altered profile of lymphocyte populations in SLE, see Linker-Israeli, (1992). Furthermore, anomalies in the surface expression of cytokine receptors in various subsets of lymphocytes, may moderate the above cellular disturbances.
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B Lymphocytes

An increased number of antibody-producing B lymphocytes is observed in SLE patients compared to normals and correlates directly with disease activity (Blaese et al., 1980), (Sakane et al., 1988). Hypergammaglobulinaemia is a characteristic feature of SLE and is in part a consequence of elevated levels of activated B cells. Many factors are involved in the cause of B cell hyperactivity. Environmental and genetic factors, together with functional defects in auto-reactive B cells, have all been linked to B cell disturbances and the production of pathogenic auto-antibodies.

The presence of a variety of auto-antibodies targeting in a non organ-specific manner implies the presence of polyclonal B cell activators, permitting antigen-independent stimulation of B lymphocytes. Increased sensitivity to cytokines promoting B cell proliferation may account for increased B cell numbers. A concomitant reduction in T lymphocyte subsets involved in suppressor activities, in response to dysregulated cytokine activity, may explain the ineffective regulation of hyperactive B lymphocytes in SLE. The presence of pathogenic specific auto-antibodies produced by IgG secreting B lymphocytes may infer cytoplasmic overexpression of auto-antigens.

Cytokines

T cell involvement is important in the regulation of balance between humoral and cell-mediated immune responses. Understandably, alterations to lymphocyte populations will significantly alter the profile of cytokine synthesis and is a characteristic feature in SLE. Resulting abnormalities in immunoregulation form the basis for cytokine involvement in the immunopathology of SLE. SLE is characterised by an overall shift towards cells supporting humoral (antibody-producing) responses and impairment of cellular immunity. This is underlined by the recognition that IL-10 levels are raised in SLE, particularly in those patients with active disease (Park et al., 1998). The anti-inflammatory nature of this cytokine serves to promote B cell proliferation while impairing cell-mediated immunity (Moore et al., 1993).
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Type 1 cytokines support cell-mediated immunity while those of Type 2 support antibody production. The cytokine profile of helper T cells is used as a means of characterisation. Consequently Th 1 cells support cell-mediated immune responses, while Th 2 cells provide help for antibody production and suppress cell-mediated responses. TNF-α is produced by Th1 cells, B cells, natural killer cells (NK) and macrophages. TNF-β is produced by activated lymphocytes. The genes encoding both cytokines are localised in close proximity within the major histocompatibility complex (MHC). TNF-α production in vitro by mitogen stimulated peripheral blood mononuclear cells (PBMCs) has been shown to vary according to the HLA class II make-up of the donor. A decreased production is observed in macrophages and lymphocytes from HLA-DR2 and DQw1 donors, while elevated levels have been observed in HLA-DR3 and DR4 donors (Jacob et al., 1990). The DR2 and DQw1 haplotype is associated with lupus nephritis. Conversely DR3 and DR4 individuals negatively correlate with lupus nephritis. It is interesting to note that TNF-α therapy has been shown to have a protective effect in B/W mice against renal pathology (Gordon et al., 1989).

IFN-γ serum levels are elevated in SLE (Funauchi et al., 1991), (al-Janadi et al., 1993), however inhibition of B and T cell proliferation is not observed. The number of cells spontaneously producing this cytokine are reportedly decreased. This feature appears to be most pronounced with increasing disease severity. A decrease in CD8+ T cell numbers may account for reduced IFN-γ production leading to impairment of macrophage and NK cell-mediated cytotoxicity.

Interleukin-1 (IL-1) production by accessory cells is decreased in most SLE patients, however monocytes spontaneously produce elevated levels of IL-1 (Sierakowski et al., 1987). Activation of T cells responding to IL-1 is diminished, even after addition of exogenous IL-1 in vitro. This suggests a possible defect at the level of the IL-1 receptor on T cells (Alcocer-Varela et al., 1984).

Interleukin-2 (IL-2) is produced by CD4+ and CD8+ T cells. Production of this cytokine in mitogen-stimulated PBMCs from SLE patients may be normal or decreased (Alcocer-Varela and Alarcon-Segovia, 1982). In some cases decreased IL-2 production has been observed to correlate with an increase in disease activity. The addition of exogenous IL-2 is not sufficient to restore T cell proliferation and
suppressor activity. However this may be overcome by *in vitro* treatment of SLE accessory cells with both IL-2 and IFN-γ. Serum IL-2 levels have been reported to be increased in some SLE patients. Variations within both clinical and experimental data, require an understanding of whether significance is to be attached to levels of IL-2 or functional activity. IL-2 responsiveness in CD4+ T cells is reduced in SLE. This may in part be due to the observed aberrations in IL-2 receptor synthesis in these cells. The differential pattern of synthesis of IL-2 receptor subunits in SLE, gives rise to a heterogeneity of possible receptor affinities. Reduced responsiveness in CD4+ T cells to IL-2 may therefore reflect a sub-population expressing low-affinity receptors (Alcocer-Varela and Alarcon-Segovia, 1982).

Interleukin-6 (IL-6) levels have been reported to be elevated in SLE (Linker-Israeli et al., 1991) and may contribute to B cell hyperactivity in this disease. Thus, cultured PBMCs from patients with SLE, have been demonstrated to spontaneously produce increased amounts of IL-6 (Klashman et al., 1991) and neutralising anti-IL-6 antibodies decrease immunoglobulin production (Linker-Israeli et al., 1991). Studies on the synthesis of IL-6 in patients with SLE, have identified B cells T cells and macrophages as the major cell types to synthesise this cytokine (Hashimoto, 1990). A role for IL-6 in the pathogenesis of SLE is further supported by the observation that levels of IL-6 in SLE correlated with disease activity (Linker-Israeli et al., 1991), (Spronk et al., 1992).

Elevated circulating levels of interleukin-10 (IL-10) have been reported in patients with SLE, being highest in patients with active disease (Park et al., 1998). A pathogenic role for IL-10 in SLE has been supported by a number of studies. PBMCs from untreated patients with SLE spontaneously release large amounts of IL-10 *in vitro* (Linker-Israeli, 1992). Furthermore, spontaneous immunoglobulin production (particularly DNA antibodies) in PBMC from patients with SLE is increased in response to IL-10 and inhibited in response to a neutralising anti-IL-10 antibody (Llorente et al., 1995). Defects in the regulation of autoreactive B and T lymphocytes by a process known as programmed cell death (apoptosis) may play a pathogenic role in SLE. It has been shown that IL-10 increases Bcl-2 expression by germinal centre B lymphocytes (Mehrian et al., 1998), preventing their death and suggesting that the stimulatory effect of IL-10 on immunoglobulin production may be partly due to
increased B lymphocyte survival. There is also evidence that disease severity in patients with SLE correlates with an increased ratio of IL-10 secreting PBMC (Hagiwara et al., 1996). A role for IL-10 in murine SLE is supported by the observation that administration of anti-IL-10 antibodies to NZB/W F1 lupus-prone mice, delays the onset of autoimmunity and production of autoantibodies (Ishida et al., 1994).

Apoptosis

Defects in apoptosis have been described in experimental models of SLE. Mutation of the membrane receptor Fas in MRL/lpr-lpr mice (which is described later), leads to decreased apoptosis and accelerated autoimmunity (Theofilopoulos and Dixon, 1985), (Nagata and Golstein, 1995). In human SLE the study of programmed cell death is an active area of research. This has been prompted by an initial claim that 60% of SLE patients have elevated levels of a functionally abnormal soluble form of the Fas receptor, which may inhibit apoptosis in lymphocytes (Cheng et al., 1994). In this study, injection of a murine homologue to the human soluble Fas protein in normal mice led to a two to three-fold increase in splenic B and T cells as well as an increase in double negative CD4- CD8- and single positive CD4+ CD8+ T cells. Furthermore it was reported that the injection of soluble murine Fas in CD1 mice led to induction of autoimmunity.

However, the majority of studies now conclude that there are no Fas abnormalities of any significance in patients with SLE. Indeed, current studies suggest that there is no problem with apoptosis per se, but rather the impaired action of macrophages in the clearance of apoptotic material. Hence, such material is thought to be a source for autoantigens which induce and maintain autoimmune responses in patients with SLE (Herrmann et al., 1998).

Elevated transcription of a proto-oncogene, Bcl-2, may also contribute to autoimmunity in human SLE. Bcl-2 is found on the inner membranes of mitochondria, the endoplasmic reticulum and the nuclear membrane and is involved in the maintenance and development of adult tissue by preventing apoptosis in
specific cell types (Korsmeyer, 1992). Consistent with this role, Bcl-2 is found in long-lived B lymphocytes within the follicular mantle zone. Overexpression of Bcl-2 in CD4+ and CD8+ T cells from SLE patients, but not B cells, has been reported as well as in a subpopulation of CD3+ CD4- CD8- T cells. Furthermore levels of Bcl-2 in these cell populations have been shown to correlate with disease activity (Aringer et al., 1994).

Autoantibodies

SLE is characterised by the presence of a wide variety of circulating antibodies directed against self antigens. Antibodies to nuclear, cytoplasmic and plasma membrane antigens have been identified and these may provide markers for disease or play a role in tissue damage (see Section 1.2).

Antibodies to nuclear antigens include those with specificity for Sm and U1RNP (Sharp et al., 1971). The former are found in 30% of black patients with SLE but only 10% of white patients. Antibodies to Ro and La are linked to the neonatal lupus syndrome (skin rashes and heart-block in the new born) (Lee and Weston, 1984). These antibodies are also associated with Sjogren's syndrome secondary to a diagnosis of SLE. Autoantibodies to Clq have been reported and correlate with glomerulonephritis (Song et al., 2001).

Antibodies to double-stranded (ds) DNA are mainly of the IgG isotype and a sub-population appears to be involved in tissue destruction. Deposition of IgG and complement has been identified in kidney biopsies from patients with glomerulonephritis, which have resulted from a previous focal immune response leading to inflammation. Eluates from affected kidneys show IgG molecules to have specificity and high affinity for ds DNA (Saito et al., 1994).

It is not clear at present which mechanisms account for the localisation of these antibodies. Glomerular localisation may result from deposition of circulating antibody-DNA complexes, leading to activation of complement and resulting in local inflammation. Alternatively a mechanism of cross-reactivity may take place in which non-complexed antibodies with specificity for DNA, interact with components of the
Introduction

glomerular basement membrane. In support of this hypothesis, heparan-sulphate, a major constituent of the glomerular basement membrane, is known to contribute an overall negative charge to the membrane. Antigen non-specific binding may therefore take place involving cationic anti-DNA antibodies. Furthermore heparin has been shown to inhibit DNA binding of antibodies eluted from the kidneys of humans and MRL-lpr/lpr mice. In the MRL-lpr/lpr model, administration of low, non-anticoagulant doses of heparin, prevents development of nephritis in SLE (Naparstek et al., 1990). Antibody-DNA complexes have in some cases been shown to contain histones. Histones possess high affinity for heparan-sulphate (Termaat et al., 1990) and antibodies to histones feature in SLE.

Experimental Models of SLE

Ethical considerations clearly limit attempts to explore the process of pathogenesis in SLE (and other diseases). To help understand the mechanisms that give rise to clinical features, animal models have been developed, the majority of which are murine (Theofilopoulos and Dixon, 1985), dogs may also spontaneously develop SLE (Theofilopoulos and Dixon, 1985). These have aided in our understanding of the immunology of the disease. Furthermore, information gathered from animal models of SLE has contributed to the understanding of the genetic factors involved in the autoimmune response in this disease. From these studies, it is clear that multiple genes are involved in the clinical and immunological manifestations of this disease. The main features of the principle murine models of SLE are shown in Table 1.3.3.

Studies of lupus-prone mice strains have shown that at least three separate genes are significant in affecting disease pathology. The MRL+/- strain spontaneously develops late-onset lupus, especially in females. Clinical nephritis develops with increasing age and death occurs by 24 months with a 50% survival time of 18 months (Theofilopoulos and Dixon, 1981). The introduction of the lpr (lymphoproliferation) gene, which includes an endogenous retroviral DNA sequence integrated into the Fas gene, leads to the expression of a dysfunctional Fas membrane receptor and leads to a loss of apoptosis. This mutation accelerates the disease.
### Table 1.3.3 Features of Lupus-Prone Mouse Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>H-2 Haplotype</th>
<th>Sex</th>
<th>In Which Disease Is Most Severe</th>
<th>Clinical Features</th>
<th>Immunological Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRL +/+</td>
<td>H-2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Female</td>
<td>Lymphoproliferation</td>
<td>Anti-nuclear antibodies, ICnephritis, rheumatoid arthritis, vasculitis, generalised lymphocyte dysfunction.</td>
<td></td>
</tr>
<tr>
<td>MRL&lt;sup&gt;-lpr/lpr&lt;/sup&gt;</td>
<td>H-2&lt;sup&gt;k&lt;/sup&gt;</td>
<td>Female</td>
<td>Same as MRL +/+ but more severe.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZB</td>
<td>H-2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Male and Female</td>
<td>Haemolytic anaemia, glomerulonephritis, Erythrocyte lymphomas</td>
<td>Anti-nuclear antibodies, IgM hyper-antibody production, generalised lymphocyte dysfunction.</td>
<td></td>
</tr>
<tr>
<td>NZB/WF&lt;sub&gt;1&lt;/sub&gt;</td>
<td>H-2&lt;sup&gt;d/z&lt;/sup&gt;</td>
<td>Female</td>
<td>Severe IC nephritis.</td>
<td>Anti-nuclear, anti-DNA antibodies.</td>
<td></td>
</tr>
<tr>
<td>BXSB</td>
<td>H-2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Male</td>
<td>Haemolytic anaemia, Glomerulonephritis, Lymphadenopathy</td>
<td>Anti-DNA antibodies, anti-thymocyte antibodies.</td>
<td></td>
</tr>
</tbody>
</table>
process such that the 50% survival time is reduced to two to four months, with 90% death occurring by nine months. An overall amplification of antibodies from the parent MRL+/- strain is attributed to lymphoproliferation, with an increase in CD4+ T cell help (Cohen et al., 1986). The introduction of the gld (generalised lymphoproliferative disease) gene into non-autoimmune C3H/HeJ mice results in lymphoproliferation and development of autoimmunity (Takahashi et al., 1994). In NZB/W F1 mice the X-linked recessive gene, xid, is observed to protect against autoimmunity through prevention of terminal B cell differentiation and the formation of autoantibody-producing B cells.

Summary

The clinical expression of SLE is the consequence of its complex immunopathology, a combination of genetic, hormonal and environmental factors. The interaction of these factors leads to the production of pathogenic autoantibodies and the formation of immune complexes. Inappropriate regulation of cell mediated immune responses, leads to ineffective clearance of autoantibody and immune complexes and possible widespread tissue and organ damage.

A growing number of studies have reported elevated levels of anti-Hsp antibodies in a number of human autoimmune diseases, including SLE. As will be discussed in the following section, anti-Hsp responses may be involved in the pathogenesis of autoimmunity.
Introduction

Section 1.4: Heat Shock Proteins In Systemic Lupus Erythematosus

Introduction

A role for heat shock proteins (Hsps) both in the normal immune system and in the aetiopathogenesis of autoimmunity, has been discussed earlier in this chapter (see Section 1.2). These studies have focussed particularly on a possible role for Hsp 60 and Hsp 70 family proteins of mycobacteria and other infectious organisms, in the pathogenesis of autoimmune diseases such as rheumatoid arthritis and vasculitis. More recently, a number of studies in both humans with systemic lupus erythematosus (SLE) and animal models of SLE, have suggested a role for Hsp 90 in the pathogenesis of this disease.

The Expression of Heat Shock Proteins In SLE

A role for Hsp 90 in the conformational regulation of steroid hormone receptors (see Section 1.1), has prompted a study of the effect of corticosteroid treatment on Hsp 90 levels in patients with SLE. Levels of Hsp 90 in peripheral blood mononuclear cells (PBMCs) from 60 randomly selected patients with SLE, were found not to correlate with corticosteroid treatment. However, levels of Hsp 90 were found to be highest in patients with active SLE compared to patients with inactive disease, healthy controls and patients with rheumatoid arthritis (Norton et al., 1989). In another study of Hsps in SLE, elevated levels of both Hsp 90 and Hsp 70 were observed in pooled peripheral blood mononuclear cells from 20 patients with SLE, compared with healthy controls (Deguchi et al., 1987). In agreement with these observations, a larger study of 102 patients with SLE showed significantly increased levels of Hsp 90 and Hsp 72 (the heat-inducible form of Hsp 70), compared with healthy controls. Importantly, for individual patients, this was not paralleled by
Introduction
elevation of the heat-inducible protein Hsp 65 or the constitutively expressed protein Hsp 73 (Dhillon et al., 1993a). The specificity of Hsp 90 and Hsp 72 elevation is further underlined by the observation that transcription of the gene encoding the Hsp ubiquitin (described in Section 1.1), is also unchanged in SLE (Twomey et al., 1992). Thus, the increased expression of Hsp 90 and Hsp 72 in SLE occurs in the absence of elevation of other Hsp family members and therefore would not appear to result from a general cellular response to stress, which would involve the increased expression of a wide range of Hsp family members.

It has been reported that approximately 30% of patients with SLE have elevated levels of Hsp 90 compared to normals (Dhillon et al., 1993a). In this study, only 10% of patients were found to have elevated levels of Hsp72. Furthermore, levels of Hsp 72 did not correlate with levels of Hsp 90, in individual patients. Thus, it appears that the elevation of these Hsps occurs as a result of distinct cellular processes within different sub-groups of patients with SLE. A genetic origin to Hsp 90 overexpression is suggested by the observation of significantly higher levels of Hsp 90 in PBMCs from patients with SLE who are HLA A1, B8, DR3 negative, compared to those who possess this haplotype (Dhillon et al., 1994). This haplotype is that most often found in caucasians with lupus. Since this ethnic group comprised 60% of the study by Dhillon et al (1993a), the reported figure of 30% for patients with SLE overexpressing Hsp 90, may be understood in relation to the genetic background of the patients in this study.

Overexpression of Hsp 90 in SLE, has been shown to be dependent on the enhanced transcription of the Hsp 90β gene, one of two separately coded isoforms of Hsp 90 found in mammalian cells (Twomey et al., 1993). In contrast, elevation of Hsp 72 is not dependent on enhanced transcription of the Hsp 72 gene and elevated levels of Hsp 72 mRNA were not observed. Post-transcriptional mechanisms which may include improved translatability of Hsp 72 mRNA, could account for the observed elevated protein levels of Hsp 72 in a subset of patients with SLE (Twomey et al., 1993). Any mechanism which is involved in Hsp 90 overexpression, occurs in a subset of patients with SLE and is a process distinct from that which leads to the general induction of Hsps in response to cellular stress.
Genetic changes to regulatory sequences in the Hsp 90β promoter may account for the enhanced transcription of this gene, which is observed in SLE (Twomey et al., 1993). Such polymorphisms might also be found within the gene encoding a cellular transcription factor, leading to activation of the Hsp 90β promoter with increased efficiency. Alternatively, differences in transcription of the Hsp 90β gene in SLE may not be due to genetic changes of this type, but may occur as part of a normal mechanism of Hsp 90 gene regulation in cells, however one in which the regulatory mechanisms are overactive in response to cellular events specific to SLE. Since Hsp 90 is known to be induced by a number of stimuli both under physiological and stressful conditions, abnormalities of such inducers in patients with SLE is a consideration. Previous studies have highlighted cytokine abnormalities in lupus (Linker-Israeli, 1992), (Kirou and Crow, 1999). Interleukin-6 (Linker-Israeli et al., 1991) and interleukin-10 (Park et al., 1998) are observed to be elevated in SLE and particularly in those patients with active disease. Moreover, previous studies which have demonstrated that interleukin-6 (IL-6) can activate the Hsp 90β promoter in vitro (Stephanou et al., 1997), suggest a role for IL-6 in the regulation of Hsp 90 expression in the normal immune system. Thus it is conceivable that in SLE, elevated levels of circulating IL-6, may significantly contribute to the observed overexpression of Hsp 90 in this disease. A similar role for interleukin-10 (IL-10), which activates target genes using a similar signalling mechanism to IL-6, in the regulation of Hsp 90 gene expression, is also possible (see Section 1.1).

Studies of Hsp expression in other autoimmune diseases, such as rheumatoid arthritis and Sjogren’s syndrome, show the pattern of Hsp 90 and Hsp 72 overexpression to be specific to SLE. Furthermore, there may be a role for Hsp 90 in distinct, clinically active patients with SLE (Dhillon et al., 1993a). Of those patients in this study overexpressing Hsp 90, patients with active neuropsychiatric or cardiovascular disease were most likely to have elevated levels of Hsp 90. A comparison of Hsp 90 expression with the presence or absence of lupus autoantibodies has shown significant elevation of Hsp 90 in patients with lupus anticoagulant and/or anti-cardiolipin antibodies (Dhillon et al., 1994). It is conceivable that Hsp 90 overexpression and the presence of these antibodies may have a common genetic origin. Also possible is a mechanism in which the expression of these
autoantibodies is provoked in response to Hsp 90 epitopes which mimic those specifically recognised by these antibodies. In agreement with this hypothesis, acute thrombocytopenia, which is associated with antiphospholipid antibodies (Harris et al., 1985) correlated with Hsp 90 overexpression (Dhillon et al., 1994).

**Surface Expression Of Hsp 90 in SLE**

A number of studies have suggested a role for heat shock proteins as autoantigens in the pathogenesis of autoimmune disease (discussed in Section 1.2). For example, mycobacterial Hsp 65 may play a role in adjuvant arthritis in rats and increased levels of antibodies to Hsp 65 have been reported in patients with rheumatoid arthritis (Tsoulfa et al., 1989). Such a role for elevated levels of Hsp 90 in provoking an autoimmune response in SLE, is likely to require the localisation of Hsp 90 on the cell surface, providing accessibility to the immune system. Relocalisation of Hsp 90 to the cell surface is a recognised phenomenon during herpes simplex virus infection (La Thangue and Latchman, 1988), in human tumour cells (Ferrarini et al., 1992) and following mitogen activation of lymphocytes (Haire et al., 1988). Surface expression of Hsp 90 in peripheral blood mononuclear cells from patients with SLE has been reported (Erkeller-Yuksel et al., 1992). As determined by flow-cytometry, surface localisation was specific to Hsp 90 and was not observed for Hsp 70 or Hsp 60 family members. Furthermore, the degree of surface Hsp 90 expression correlated with the degree of Hsp 90 overexpression.

**Antibodies to Hsps in SLE**

Autoantibodies to Hsp 90 (Conroy et al., 1994) have been detected in the sera of patients with SLE and other autoimmune diseases. Previous studies (Minota et al., 1988), (Winfield and Jarjour, 1991), had produced conflicting data on the incidence of antibodies to Hsp 90 in SLE compared to normal individuals and subjects with other autoimmune diseases. However, using an ELISA system to detect antibodies to the native form of Hsp 90, rather than the reduced form used in the report by (Minota
et al., 1988), a study of 73 patients with SLE has shown raised IgG antibodies in 26% of subjects and raised IgM antibodies in 35% of the patients, compared to healthy normal individuals (Conroy et al., 1994). Patients with raised anti-Hsp 90 antibodies were most likely to lack the HLA A1/B8/DR3 haplotype. The absence of this haplotype was previously reported to correlate with overexpression of Hsp 90 (Dhillon et al., 1994). In contrast, elevated levels of antibodies to Hsp 70 were rarely found in individual patients with SLE.

**Hsp 90 Expression in MRL/lpr-lpr Mice**

Evidence of a primary genetic origin to Hsp 90 overexpression in SLE is supported by studies in autoimmune MRL/lpr-lpr mice which develop an age-dependent lupus-like disease involving the production of high levels of double-stranded DNA antibodies. Clearly, ethical considerations limit the study of lupus in humans prior to disease onset. Thus, investigations of the pathogenesis of autoimmunity in MRL/lpr-lpr mice are clearly of interest. The primary genetic defect in this strain is the inactivation of the gene which encodes the Apo-1/Fas antigen which mediates apoptosis (Watanabe-Fukunaga et al., 1992).

A study of Hsp 90 levels in various tissues in MRL/lpr-lpr mice has been reported (Faulds et al., 1994). Levels of Hsp 90 were found to be elevated in the spleen of MRL/lpr-lpr mice compared to MRL++ mice (which develops a milder version of the disease) and BALB/C mice, while an analysis of various other tissues including the kidney and liver, showed no such elevation. Elevated levels of Hsp 90 in the spleen of these mice occurred independent of age and preceded the onset of disease and antibodies to Hsp 90, suggesting a possible role for Hsp 90 in the development of autoimmunity. In contrast, elevated levels of Hsp 72 in MRL/lpr-lpr mice, were found in spleen and liver tissue, compared to BALB/C mice. This elevation was age-dependent and increased with disease progression, possibly as a response to cellular stress. The observed elevation of Hsp 90 in MRL/lpr-lpr mice was not paralleled by elevation of Hsp 73 or Hsp 60. The specific elevation of Hsp 90 in these mice (preceding the onset of disease) in the absence of elevation of other Hsps, parallels observations in human lupus (Dhillon et al., 1993a).
Taken together, the studies outlined in this section suggest a link between Hsp 90 overexpression in SLE and the development of anti-Hsp 90 antibodies in this disease, with possible pathological consequences (see Figure 1.4.1). Any role for Hsp 90 overexpression in the pathogenesis of SLE, must involve specific environmental factors which regulate Hsp 90 gene expression. Furthermore, previous observations which highlight a genetic origin to Hsp 90 overexpression and the development of ant-Hsp 90 antibodies are consistent with a role for susceptibility genes in the development of SLE, which is discussed in Chapter 1, Section 1.3.
Figure 1.4.1 illustrates a hypothetical model for autoimmune pathology in SLE. An involvement of genetic and environmental factors leads to the overexpression and cell surface localisation of Hsps, with possible pathological consequences.
Introduction

Section 1.5: Aims of Dissertation

It is clear from the studies presented in Sections 1.1 to 1.4, that the regulation of heat shock proteins (Hsps) within the cell is very finely tuned and occurs differentially in patients with systemic lupus erythematosus (SLE). Previous studies have associated the expression of the 90 kDa heat shock protein (Hsp 90) with active clinical subsets in SLE. Moreover, a role for Hsp 90 in the pathogenesis of SLE may involve the induction of antibodies to this protein. However, the mechanisms by which Hsp 90 is overexpressed in SLE are unclear and a link between this event and the induction of antibodies to Hsp 90 has not been established. A role for cytokines in the regulation of Hsps has been discussed in Section 1.1 and Section 1.4. Thus, interleukin-6 has already been shown to induce Hsp 90 expression and is known to be elevated in SLE. Taken together, these studies suggest that elevated levels of cytokines in SLE, may lead to the overexpression of Hsp 90 in SLE and the subsequent induction of antibodies to Hsp 90, with possible pathological consequences.

Outline of Investigations

1. The effect of the cytokine interleukin-10 (which is elevated in SLE) on Hsp 90 levels in different cells was investigated.

2. The relative effect of interleukin-10 and/or interleukin-6 on Hsp 90 levels in SLE, was studied by the quantitative determination and subsequent correlation, of the levels of Hsp 90 and cytokines in patients with this disease.

3. A role for Hsp 90 overexpression in the development of antibodies to Hsp 90 in SLE, was investigated by the quantitative determination of antibodies to Hsp 90 and subsequent correlation with levels of Hsp 90 in the same patients.
4. Finally, associations of levels of cytokines, Hsps and antibodies to Hsps, with active disease in SLE, were studied using the BILAG index. Thus, the relative contribution of these variables to pathogenesis in SLE, was established in patients graded by active clinical subsets of this disease.
Chapter 2
Materials and Methods

Section 2.1: Analysis of Hsp 90 Gene Expression in Cultured Cells

Materials

Laboratory Chemicals

General laboratory chemicals were of analytical grade and were purchased from either Sigma Chemical Company, Poole, Dorset, U.K, from Boehringer Mannheim, Lewes, East Sussex, U.K or from Merck Ltd., Poole, Dorset, U.K. Other reagents and glassware were purchased from the central supplies of the Medical Molecular Biology Unit.

Monoclonal Antibodies

Mouse monoclonal antibody AC88 was a gift from Professor D.O. Toft, Mayo Clinic Foundation, Rochester, Minnessota, USA. Antibody AC88 is raised in mice and is of class IgG\(_1\). AC88 was developed following isolation of Hsp 90 from the water mould Achyla ambisexualis and has been shown to react with human Hsp 90 (Riehl et al., 1985). AC88 also reacts with a number of 25-40 kDa proteins which have been identified as heterogenous ribonuclear proteins (Minoo et al., 1989). Mouse monoclonal antibody N27F3-4 (class IgG\(_1\)) was purchased from Stressgen Biotechnologies Inc., Canada and reacts with human Hsp 73 and displays faint reactivity with human Hsp 72. This was confirmed in the study of Dhillon, (1993b).
Methods

Cytokines

Recombinant human interleukin-10, interleukin-6 and interferon-γ, were each purchased from Santa Cruz Biotechnology, Inc., USA. Each cytokine was provided as a 5 mg sample of lyophilised protein. This was subsequently resuspended in 1 ml of sterile distilled water and stored at -20°C, as single-use aliquots until required.

Cells and Cell Lines

The HepG2 Cell Line

The human hepatoma cell line HepG2, stably expressing the human IL-10 receptor (Lai et al., 1996), was a kind gift from Dr. Heinz Baumann, Roswell Park Cancer Institute, Buffalo, NY, USA.

Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinised venous blood. Approximately 20 ml of blood was drawn from each normal individual and this would yield on average 20 million cells for subsequent culture. Purification of PBMCs is detailed later.

Plasmids

The 5' Hsp 90β promoter chloramphenicol acetyl transferase (CAT) reporter construct Hsp 90β-CAT (Rebbe et al., 1987) was a kind gift from Dr. Neil Rebbe (Washington University School of Medicine, St. Louis, MO). Construct Hsp 90β-CAT consists of a fragment -1044 to +36 relative to the transcriptional start site, coupled to a CAT vector. The 5' Hsp 90α promoter CAT reporter construct Hsp 90α-CAT, was a kind gift from Dr. Lee Webber. Construct Hsp 90α-CAT consists of a fragment -1050 to +42 relative to the transcriptional start site, coupled to a CAT vector.

The Hsp 90β promoter wild-type and mutant STAT-3 vectors have been previously described (Stephanou et al., 1998b). Construct Hsp 90-S3E consists of a sequence of the Hsp 90β promoter from -643 to -623 relative to the transcriptional start site. Construct Hsp90-MS3E is identical to Hsp 90-S3E except for adenine residues at position -641 and -631, replacing the wild type cytosine residues. Both the wild-type
Methods

and mutant Hsp 90β promoter STAT-3 sequences (see Figure 2.1) are cloned upstream of the thymidine kinase promoter and the CAT reporter gene in the vector pBLCAT2.

The wild-type STAT-3 expression vector contains the full coding region for STAT-3, cloned into the mammalian expression vector pEF-Bos and expressed under control of the elongation factor gene promoter (Minami et al., 1996).

Enzymes

Restriction enzymes and enzyme buffers were purchased from Promega, Southampton, U.K or from Life Technologies, Paisley, Renfrewshire, U.K.

Methods

Isolation of PBMCs

Fresh heparinised venous blood was diluted 1:1 with Roswell Park Memorial Institute medium (RPMI-1640) (Sigma, Dorset, U.K.). 20 ml aliquots of this mixture were layered onto 15 ml Ficoll-Pacque (Pharmacia, UK.) in Falcon tubes (Greiner, Germany). These samples were centrifuged at 800 x g for 30 mn at room temperature. The resulting buffy coat, containing PBMCs was transferred to a fresh falcon tube. PBMC obtained by Ficoll density centrifugation were pelleted at 3000 x g for 10 mn. at room temperature, washed once with PBS and pelleted in a microfuge at 13,000 x g for 1 mn at room temperature and stored at −70°c.

Cell Culture

HepG2 Cells

HepG2 Cells were maintained in monolayer cultures in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) bovine foetal serum (Life Technologies, Inc.) at 37°C in a 5% CO₂ incubator. For each experiment, cells were trypsinised and re-plated at a density of 10⁶ per well in DMEM plus 10% (v/v) foetal bovine serum in six-well plates (Nunc, Uxbridge, U.K) overnight.
Figure 2.1  Hsp 90 Promoter Sequences in pBLCAT2

Synthetic Oligonucleotides

(a) Hsp90-S3E

\[
\begin{align*}
\text{STAT-3} & \quad \text{STAT-3} \\
\text{GCCTGGAAACTGCTGGAAAT} & \\
\text{HSE} & \quad \text{HSE} & \quad \text{HSE}
\end{align*}
\]

(b) Hsp90-MS3E

\[
\begin{align*}
\text{GCATGGAAACTGATGGAAAT} \\
\text{STAT-3 Consensus Sequence} & \quad CTGGRAA
\end{align*}
\]

Figure 2.1 shows synthetic oligonucleotides corresponding to the wild-type Hsp 90β promoter STAT-3 binding site (a) and a mutant derivative (b), which were cloned upstream of the thymidine kinase promoter and the CAT reporter gene in the vector pBLCAT2.
Methods

PBMCs

PBMCs were cultured at 10^6 cells per well in RPMI-1640 medium plus 10% (v/v) bovine foetal serum (Life Technologies, Inc.) in six-well plates (Nunc, Uxbridge, U.K) at 37°C in a 5% CO₂ incubator.

Stimulation

Following plating, HepG2 cells or PBMCs were maintained in their appropriate complete medium over night, then washed twice with PBS and subsequently cultured in complete medium containing 1% (v/v) bovine foetal serum (Life Technologies, Inc.) for a further 6 hr before addition of cytokines at the concentrations indicated in the figure legends in Chapter 3.

Analysis of Heat Shock Protein Levels in Cultured Cells

Preparation Of Whole Cell Lysate

Cells were removed from six-well plates using a rubber policeman and pelleted in a microfuge at 13,000 x g for 1 mn. The resulting pellet was resuspended in ice cold 0.1 M Tris (pH 7.5) and subjected to three cycles of freeze-thaw in liquid nitrogen and the lysate pelleted at 4°C in a microfuge for 40 mn at 13,000 x g. The resulting clear lysate was removed and stored at −70°C.

Protein Electrophoresis And Western Blotting

Following treatment with cytokines, cells were harvested as described above for Western blot analysis which was performed as described previously (Stephanou et al., 1997). Whole cell lysate samples were added in a 1:1 ratio to sample buffer containing 2.3% (w/v) sodium dodecyl sulphate (SDS), 0.0625 M Tris, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and bromophenol blue, at a concentration of 10^6 cells to 50 μl of sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), according to the method of Laemmli., (1970). A vertical, one-dimensional electrophoresis system was used (Gibco-BRL), Paisley, Scotland. The lower resolving gel consisted of 7% (w/v) acrylamide (in 1.5 M Tris, 0.4% w/v SDS, pH 8.8) and the stacking gel consisted of 5% (w/v) acrylamide (in 0.5 M Tris, 0.4% w/v SDS, pH 6.8).
Methods

Protein samples were loaded and electrophoresed in duplicate on separate gels, in running buffer (0.192 M glycine, 0.025 M Tris, 0.1% w/v SDS, pH 8.3), for 4 hr at 40 mA at room temperature. Both gels were used for Western blotting onto nitrocellulose Hybond C (Amersham, Buckinghamshire, UK). Transfer was by electrophoresis in a blotting tank (Gibco-BRL), using blotting buffer of 0.192 M glycine, 0.025 M Tris, 20% (v/v) methanol, (pH 8.0) for 12 hr at 200 mA and at 4°c.

Development Of Western Blots

Western blots were initially blocked with 4% (w/v) Marvel (Nestle, U.K.), phosphate-buffered saline (PBS) and 0.05% (v/v) Tween-20 for 1 hr at room temperature and washed 3 times in 0.3% Marvel, PBS, 0.05% Tween-20, and once in PBS, for 5 min each wash. Blots were then incubated with a primary antibody, washed as above and subsequently a secondary antibody conjugated to horseradish peroxidase, using the conditions outlined in Table 2.1. Blots were then washed as described above and were developed (see Figure 2.2) using an enhanced chemiluminescence (ECL) kit (Amersham, U.K.), with autoradiograph exposures of 5 s to 1 min at room temperature.

Bacterial Strains and Growth Conditions

Bacterial Strains

Escherichia coli (E.coli)

DH5α: supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1
GyrA96 thi-1 relA1 (Bethesda Research Labs, 1986)

Luria Bertani (LB) media

1% (w/v) Bacto™-tryptone
1% (w/v) NaCl
0.5% (w/v) Bacto™-yeast extract

LB Agar

LB media (prepared as above) containing 2% (w/v) Bacto™-agar.
Table 2.1   Antibody Conditions

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Conditions for Hybridisation</th>
<th>Antibody Dilution</th>
<th>Secondary Antibody Conditions</th>
<th>Protein Detected</th>
<th>Primary Antibody Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC88</td>
<td>2 hr at room temperature</td>
<td>1:500</td>
<td>1:1000 dilution of HRP-anti mouse IgG (Dakopatts, Denmark)</td>
<td>Hsp 90 (90 kDa)</td>
<td>David Toft, Mayo Clinic Foundation, Rochester, Minnesota, USA</td>
</tr>
<tr>
<td>N27F3-4</td>
<td>2 hr at room temperature</td>
<td>1:1000</td>
<td>1:1000 dilution of HRP-anti mouse IgG (Dakopatts, Denmark)</td>
<td>Hsp 70 (70 kDa)</td>
<td>Stressgen Biotechnologies Inc., Canada</td>
</tr>
<tr>
<td>Actin</td>
<td>1 hr at room temperature</td>
<td>1:2000</td>
<td>1:2000 dilution of HRP-anti goat IgG (Dakopatts, Denmark)</td>
<td>Actin (40 kDa)</td>
<td>Stressgen Biotechnologies Inc., Canada</td>
</tr>
</tbody>
</table>
Figure 2.2  Principle of ECL Western Blotting

(a) [Chemical reaction diagram]

(b) [Diagram showing protein binding and light emission]

Figure 2.2 shows the HRP/hydrogen peroxide catalysed reaction of luminol and the production of light (a). Following oxidation, the luminol is in an excited state which decays to a ground state via a light emitting pathway which can be detected by lightsensitive autoradiography film (b).
Methods

Media were autoclaved at 120°C for 30 min at 10 lb square/inch. Ampicillin (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) was prepared as a 100 mg/ml stock, which was filter sterilised using a 0.45 μm Sartorius filter and stored at -20 °C. Cultures of *E.coli* contained a final concentration of 0.1 mg/ml of ampicillin. Starter cultures of *E.coli* were grown in 5 ml of LB medium o/n at 37 °C in a Gallenkamp orbital shaker. Approximately 500 μl of the overnight culture was used to inoculate a 500 ml volume of medium which was subsequently incubated at 37°C o/n to the required cell turbidity. For long term storage of *E.coli* cultures, 500 μl glycerol was added to 500 μl of an overnight culture and stored at -70°C. Alternatively, cultures were streaked onto LB Agar (prepared as above), incubated overnight at 37°C and stored at 4°C for up to 3 months.

Transformation of *E.coli*.

This method is adapted from Hanahan, (1985). *E.coli* is rendered competent to take up DNA by the treatment of cells with MgSO^4_4 and MgCl_2. Divalent cations (Mg^{2+}) are thought to modify channels in the cell membrane thereby allowing DNA to enter cells.

An overnight culture of *E.coli* cells (DH5α) was prepared as described earlier. A loopful of cells was used to inoculate 50 ml of SOC medium, which was subsequently cultured at 37°C to an OD_{560} of 0.4-0.55 units. Cells were then pelleted by centrifugation at 2,000 g at 4°C for 10 min. The resulting pellet was resuspended in 15 ml ice cold RFB I and incubated on ice for 15 min. Cells were pelleted and resuspended in 4 ml ice cold RFB II. Aliquots were snap frozen in liquid nitrogen and stored at -70°C.

For transformation of *E.coli*, 100 μl aliquots of cells were thawed and each aliquot was added to 10 μl of ice cold 1.74% β-mercaptoethanol solution and incubated on ice for 3 mn. DNA to be transformed (approximately 100 ng) was added to the cell mix and incubated on ice for 40 mn. Cells were heat shocked at 42°C for 3 mn, cooled briefly on ice and added to 500 μl of LB medium and incubated at 37°C for 30 mn. Half of the cell mix was subsequently plated onto LB agar plates (prepared as described earlier) supplemented with 0.1 mg/ml ampicillin and incubated overnight at 37°C.
Methods

**SOB Medium:**

- 0.02% w/v Bacto-tryptone
- 0.005% w/v Bacto-yeast extract
- 10 mM NaCl
- 2 mM KCl

**SOC Medium:**

- SOB medium supplemented with 10 mM MgSO\(_4\) and 10 mM MgCl\(_2\)

**RFB I:**

- 3 mM KAc
- 5 mM MnCl\(_2\) \(\cdot \) 4H\(_2\)O
- 10 mM CaCl\(_2\)
- 100 mM RbCl
- 15% Glycerol
- 2 mM Acetic Acid

  Final pH was adjusted to 5.8.
  The resulting solution was then filter sterilised and stored at 4°C.

**RFB II:**

- 5 mM MOPS, pH 6.8
- 37.5 mM CaCl\(_2\)
- 10 mM RbCl
- 15% glycerol

  Final pH was adjusted to 5.8, the resulting solution was then filter sterilised and stored at 4°C.
Methods

Small Scale Plasmid DNA Extraction from *E. coli*

This method is an adaption of that described by Sambrook et al., (1989) for the rapid isolation and analysis of recombinant DNA from *E.coli* transformants. This method exposes cells to detergent then alkali solutions, which disrupt the DNA base pairing of and hence denature, the linear chromosomal genome. Plasmid DNA is protected from denaturation due to the supercoiled circular nature of the DNA. Denatured chromosomal DNA and proteins, can then be removed by centrifugation whereas the plasmid DNA remains in solution until precipitated with isopropanol.

A 5 ml volume of LB medium supplemented with 100 µg/ml of ampicillin, was inoculated with a single colony of *E.coli* containing the plasmid of interest and the culture grown overnight as described previously. The culture (1.5 ml) was then harvested at 13,000 x g in a microfuge and the cell pellet resuspended in 100 µl ice cold Solution I and then 200 µl of Solution II added to the mix. This was then placed on ice and 150 µl ice cold solution III added to the cell suspension which was vortexed and incubated on ice for a further 5 mn. The resulting cell debris was pelleted at 13,000 x g for 5 mn in a microfuge and the supernatant transferred to a fresh tube. A few drops of phenol:chloroform (1:1 ratio) were added to the supernatant to remove contaminating proteins. This mix was vortexed and centrifuged at 13,000 x g for 2 mn. The top aqueous layer was then transferred to a fresh tube and the DNA precipitated with two volumes of ethanol and incubated at 20°C for 30 mn and then centrifuged at 13,000 x g for 10 mn. The resulting pellet was allowed to air dry for 5 mn and was subsequently resuspended in 16 µl H₂O, 4 µl RNase A (10 mg/ml). Restriction analysis of DNA was subsequently performed as described later and transformation into *E.coli* was performed as described earlier.

**Solution I**

50 mM Glucose  
25 mM Tris (pH 8.0)  
10 mM EDTA (pH 8.0)  
Stored at 4°C
Methods

Solution II

0.2 M Na OH
1% SDS
Freshly Prepared

Solution III

5M CH₃COOK
11.5% (v/v) glacial acetic acid

Large Scale Plasmid DNA Extraction from E.coli

A single colony of transformed E.coli was used to inoculate 500 ml of LB medium supplemented with ampicillin (final concentration 100 μg/ml). This culture was grown overnight at 37°c, as described previously. Cells were harvested by centrifugation at 3,000 x g for 30 mn and the resulting cell pellet was resuspended in 10 ml ice cold Solution I (described earlier) and transferred to a fresh tube. This suspension was then gently mixed with 20 ml Solution II (described earlier) and then 15 ml Solution III (described earlier). This mix was then incubated on ice for 15 mn and then centrifuged at 3,000 x g for 15 mn. The resulting supernatant was removed, transferred to a fresh tube with an equal volume of isopropanol to precipitate DNA and centrifuged at 5,000 x g for 15 mn. The resulting pellet was resuspended in 3 ml TE (pH 8.0) and 3 ml ice cold 5 M LiCl. This solution was centrifuged at 10,000 x g for 15 mn to remove contaminating RNA and the supernatant transferred to a fresh tube. An equal volume of isopropanol was added to precipitate DNA and the mix centrifuged at 10,000 x g for 15 mn.

The resulting pellet was resuspended in 500 μl 0.1 M Tris (pH 8.0) and incubated at 37°c for 30 mn with 10 μl RNase A (10 mg/ml) to remove any further contaminating RNA. DNA was precipitated with an equal volume of PEG solution and incubated at 4°c overnight. The precipitate was pelleted by centrifugation in a microfuge at 13,000 x g for 15 mn and resuspended in 400 μl 10 mM Tris (pH 8.0), 0.5 M NaCl. Contaminating protein was removed by the addition of an equal volume of phenol, followed by vortexing and centrifugation in a microfuge at 13, 000 x g for 5 mn. The top aqueous layer was then transferred to a fresh tube and a further phenol extraction carried out. Two extractions with equal volumes of chloroform were carried out to remove any residual phenol. DNA was then precipitated with 1 ml ethanol, incubated at -
Methods

20°C for 30 min and centrifuged at 13,000 x g for 15 min in a microfuge. The resulting DNA pellet was resuspended in 100 μl ddH₂O and quantified as described below.

PEG Solution

20% polyethylene glycol 6000
1 M NaCl
10 mM Tris (pH 8.0)

Quantitative Determination of Plasmid DNA

5 μl of a plasmid DNA preparation was diluted into 1 ml of double distilled (dd) H₂O. This was subsequently quantified spectrophotometrically using the general formula:

\[ 1 \text{ A}_{260} \text{ unit} = 50 \mu g/ml \]

Restriction Analysis of DNA

Small scale digests were routinely used to confirm that plasmid DNA preparations contained the required plasmid. Digests were carried out in 20 μl volumes at 37 °C for 1 hr, using an appropriate enzyme with buffer as recommended by the manufacturers.

Digest Mix

DNA (up to 1 μg) 1 μl
Buffer (10 x) 2 μl
Enzyme (1 unit) 1 μl
ddH₂O 16 μl
Methods

Agarose Gel Electrophoresis

In general, 1% agarose gels were prepared. 1.0 g of ultra pure agarose was dissolved in 100 ml of 1 x TAE by heating in a microwave. Ethidium bromide was added to a final concentration of 0.5 mg/ml. The liquid agarose was subsequently poured into a Biometra Agagel™ gel caster and a suitable comb fitted. The agarose was allowed to solidify at room temperature, at which point the comb was removed. Samples and DNA marker, each containing 1 x loading dye, were separately loaded onto the gel. Samples were electrophoresed at 80 Volts and the gel was photographed under UV light.

1 x TAE

0.4 M Tris
0.2 M Sodium Acetate
20 mM EDTA
pH adjusted to 8.3 with HCl

10 x Loading Buffer

0.025% bromophenol blue
0.025% xylene cyanol
30% glycerol

Ethidium Bromide

10 mg/ml dissolved in ddH₂O and stored in the dark at room temperature.

DNA Markers

1 kilobase DNA ladder was purchased from Life Technologies, Paisley, Renfrewshire, U.K. and was diluted 1:5 with 1 x loading buffer and stored at −20°c.
Methods

Transfection and Stimulation of Hsp 90 Promoter CAT Constructs

Transfection of Hsp 90α and –β promoter CAT reporter constructs was performed as described previously (Stephanou et al., 1997) by the calcium phosphate method of Gorman, (1985). IL-10 responsive HepG2 cells were plated at a density of 10^6 cells/well in six-well plates. Cells were transfected as described below with Hsp 90 promoter-CAT reporter constructs, with or without a STAT-3 expression vector at the concentrations indicated in the figure legends (see Chapter 3) and 1 µg of a control β-galactosidase expression vector. To normalise for transfection efficiency, CAT activities were corrected for β-galactosidase activities. Following a period of 4-6 hr, cells were washed five times in phosphate-buffered saline (PBS) and stimulated with cytokines as described below.

2 x HEPES Buffered Saline (HBS)

274 mM NaCl
50 mM HEPES
2 mM Na_2HPO_4
pH was adjusted to 7.12 with 1 M NaOH, stored at 4°C and filter sterilised before use.

Tube A

2 M CaCl_2·6H_2O 31 µl
DNA (1-5 µg) 10 µl
ddH_2O to bring the total volume to 250 µl

Tube B

2 x HBS (pH 7.12) 250 µl

The contents of Tube A were added dropwise to Tube B with constant flicking and allowed to form a precipitate at room temperature for 45 min. This mix was subsequently added dropwise to the cells and incubated for 4-6 hr as described earlier. Cells were then washed five times with complete medium to remove any precipitate and
Methods

were maintained in complete medium for 24 hr before a 5 hr period of culturing in complete medium in which the bovine foetal serum has been previously heat-inactivated (Life Technologies, Inc.), followed by stimulation with 50 ng of IL-10 (or another cytokine at a concentration as indicated in the figure legends in Chapter 3). Cells were harvested after 15 hr of stimulation using a rubber policeman, pelleted at 15,000 x g. The resulting pellet was resuspended in 100 μl ice cold 0.25 M Tris (pH 7.5) and lysed by three cycles of repeated freeze-thawing in liquid nitrogen and centrifuged at 13,000 x g for 45 mn in a microfuge. The supernatant was transferred to a fresh tube and stored at -20 °C.

**Chloramphenicol Acetyl Transferase (CAT) Assay**

Following transfection and transcriptional activation of Hsp 90 promoter CAT constructs, the enzyme chloramphenicol acetyl transferase is produced. This enzyme is readily assayed by a determination of its activity as measured by the acetylation of a substrate [14C]-chloramphenicol. The amount of the product is determined by thin-layer chromatography (TLC), as outlined below.

**CAT Assay Reaction Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 M Tris (pH 7.5)</td>
<td>70 μl</td>
</tr>
<tr>
<td>D-Threo [dichloroacetyl-1-14C] chloramphenicol (54 mCi/mmol)</td>
<td>4 μl</td>
</tr>
<tr>
<td>4 mM acetyl Co-enzyme-A</td>
<td>20 μl</td>
</tr>
<tr>
<td>equalised volume of cell extract plus 0.25 M Tris (pH 7.5)</td>
<td>55 μl</td>
</tr>
</tbody>
</table>

Assays of CAT activity were performed on samples that had been equalised for protein content, as determined by the method of Bradford, (1976). The above reaction was incubated at 37°C for 1 hr and the chloramphenicol was extracted by addition of 1 ml of ethyl acetate. This mix was subsequently vortexed for 30 s and centrifuged at 13,000 x g for 2 mn. The top organic layer was transferred to a fresh tube and vacuum dried. Samples were resuspended in 15 μl ethyl acetate and spotted onto a silica gel TLC plate (Whatman International Ltd., Maidstone, Kent, U.K.). This plate was subjected to ascending chromatography with a 95:5 mix of chloroform:methanol, until the front had reached 1 inch from the top of the TLC plate. The plate was allowed to air
Methods

dry, before being exposed to X-Ray film and quantified by scanning densitometry using a Bio-Rad (Hemel Hempstead, U.K.) Imaging Densitometer.
Methods

Section 2.2 Quantitative Determination of Heat Shock Proteins in SLE

Levels of Hsp 90 and Hsp 70 were determined in individual samples of PBMCs, obtained from patients with SLE or RA and normal individuals, using the method of Dhillon et al (1993a; 1993b).

Materials

Monoclonal Antibodies

Monoclonal antibody AC88 to Hsp 90 has been previously described in Section 2.1 of this chapter. The mouse monoclonal antibody N27F3-4 to Hsp 70 and a goat polyclonal antibody to actin were both purchased from StressGen (Victoria, B.C., Canada).

Blood Samples

PBMCs obtained from normal individuals and patients with SLE or RA, were isolated from individual heparinised venous blood samples, as described in Section 2.1 of this chapter.

Patients and Normal Individuals

All patients with SLE fulfilled the American Rheumatism Association (ARA) 1982 revised criteria for the classification of SLE (see Chapter 1, Section 1.1). Samples of venous blood were taken from patients attending the morning outpatient SLE Clinic or the RA Clinic of the Bloomsbury Rheumatology Unit, Department of Medicine, University College London. Normal individuals consisted of healthy volunteers from The Department of Molecular Pathology, The Windeyer Institute for Medical Sciences, University College London. The age and sex distribution of patients and normal individuals is provided in Table 2.2 and the ethnic origin of patients with SLE is provided in Table 2.3.
Methods

Table 2.2 Age and Sex Distribution of Patients with SLE, RA and Normal Individuals

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean Age</th>
<th>Median Age</th>
<th>Age Range</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>116</td>
<td>36</td>
<td>38</td>
<td>18-68</td>
<td>3</td>
<td>113</td>
</tr>
<tr>
<td>RA</td>
<td>20</td>
<td>39</td>
<td>41</td>
<td>29-76</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Normals</td>
<td>30</td>
<td>29</td>
<td>26</td>
<td>21-57</td>
<td>5</td>
<td>25</td>
</tr>
</tbody>
</table>

Key:

n: Number of subjects

SLE: Systemic lupus erythematosus

RA: Rheumatoid arthritis
### Methods

Table 2.3 Ethnic Origin of Patients with SLE, RA and Normal Individuals

<table>
<thead>
<tr>
<th>Ethnic Origin</th>
<th>SLE</th>
<th>RA</th>
<th>Normals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>71</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>Afro-Carribean</td>
<td>21</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Caucasian/Afro-Carribean</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Indian</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>West Indian</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Chinese</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>116</strong></td>
<td><strong>20</strong></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>

**Key:**

SLE: Systemic lupus erythematosus

RA: Rheumatoid arthritis
Methods

Preparation of Whole Cell Lysate from PBMCs

PBMCs were purified from venous blood samples as described earlier. The resulting cell pellet was resuspended in ice cold 0.1 M Tris (pH 7.5) and subjected to three cycles of freeze-thaw in liquid nitrogen and the lysate pelleted at 4°C in a microfuge for 40 min. at 13,000 x g. The resulting clear lysate was removed and stored at -70°C.

Protein Electrophoresis And Western Blotting

Western blot analysis was performed as described previously (Stephanou et al., 1997), using whole cell lysate prepared as described above. Protein samples were added in a 1:1 ratio with sample buffer containing 2.3% (w/v) sodium dodecyl sulphate (SDS), 0.0625 M Tris, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and bromophenol blue at a concentration of 10^6 cells to 50 μl of sample buffer. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), according to the method of Laemmli., (1970). A vertical, one-dimensional electrophoresis system was used (Gibco-BRL), Paisley, Scotland). The lower resolving gel consisted of 7% (w/v) acrylamide (in 1.5 M Tris, 0.4% w/v SDS, pH 8.8) and the stacking gel consisted of 5% acrylamide (in 0.5 M Tris, 0.4% w/v SDS, pH 6.8). Protein samples were loaded and electrophoresed in duplicate on separate gels, in running buffer (0.192 M glycine, 0.025 M Tris, 0.1% w/v SDS, pH 8.3), for 4 hr at 40 mA at room temperature. Both gels were used for Western blotting onto nitrocellulose Hybond C (Amersham, Buckinghamshire, UK). Transfer was by electrophoresis in a blotting tank (Gibco-BRL), using blotting buffer of 0.192 M glycine, 0.025 M Tris, 20% methanol, (pH 8.0) for 12 hr at 200 mA at 4°C.

Development Of Western Blots

Western blots were initially blocked with 4% (w/v) Marvel, phosphate-buffered saline (PBS) and 0.05% Tween-20 for 1 hr at room temperature and washed 3 times in PBS containing 0.3% Marvel, 0.05% Tween-20, and once in PBS, for 5 min each. Blots were then incubated with a primary antibody and washed as above and subsequently a secondary antibody conjugated to horseradish peroxidase, using the conditions outlined in Table 2.1. Blots were then washed as described above and were developed (see Figure 2.2) using an enhanced chemiluminescence (ECL) kit (Amersham, U.K.), with
Methods

autoradiograph exposures of 5 s to 1 mn at room temperature. Representative autoradiographs of immunoblots for Hsp 90 (Figure 2.3), Hsp 70 (Figure 2.4) and actin (Figure 2.5) are illustrated in the respective figures. In Figure 2.3, the 25-40 kDa bands are heterogenous ribonuclear proteins, which are reactive with antibody AC88 to Hsp90 as mentioned earlier (see p.103).

Calculation of Heat Shock Protein Levels

Autoradiographs were scanned within the linear range using the VD620 densitometer (Biorad, Hemel Hempstead, U.K). Levels of Hsp were calculated by the method of Dhillon et al., (1993a; 1993b). Values for Hsp levels of individual samples, obtained by densitometric scanning, were expressed as a ratio of the value obtained for the respective Hsp of the positive control standard Jurkat T cell line, which constitutively overexpresses Hsps (Dhillon, 1993b). Actin levels remain relatively constant in cells and have been demonstrated to be a good measure of total cell protein (Preston et al., 1990). Thus, Hsp values between samples were equalised for variations in protein loading, by scanning actin bands of samples and Jurkat standard. Differences in the level of an Hsp between successive generations of Jurkat standard were equalised by calibrating each new generation against the first generation. An equalisation factor (E) was therefore obtained for successive Jurkat standard generations. The formula for calculating levels of Hsp 90 or Hsp 70 in individual samples is as follows:

\[
\frac{\text{Hsp band sample (OD x mm)}}{\text{Actin band sample (OD x mm)}} = \frac{\text{Hsp band Jurkat (OD x mm)}}{\text{Actin band Jurkat (OD x mm)}} \times E
\]

Accuracy of the Procedure for the Quantitative Determination of Hsps

Samples were sonicated for homogeneity and were loaded onto gels using a Hamilton syringe to ensure accuracy of volumes loaded. Hyperfilm-ECL (Amersham, U.K.) was used for autoradiography, which the manufacturers state produces a linear response to the light emitted by enhanced chemiluminescence. Different exposure times of autoradiographs or runs of sample over time, were assessed for intra- and inter-assay variation. The mean percentage error (mean absolute error ÷ mean Hsp 90 x 100) for any Hsp 90 value was determined as 11%, which was equivalent to that reported in the study of Dhillon (1993b) which recorded a value of 13% mean percentage error. The mean percentage error for any Hsp 70 value was determined to be 9%, however no
Figure 2.3. Representative autoradiograph of an Hsp 90 immunoblot. Samples from the Jurkat standard (Lane 1), patients with SLE (Lanes 2-9), patients with RA (Lanes 10-14) and from normal individuals (Lanes 15-16), are indicated in the figure and were subsequently analysed by densitometric scanning.
Figure 2.4. Representative autoradiograph of an Hsp 70 immunoblot. Samples from the Jurkat standard (Lane 1), patients with SLE (Lanes 2-9), patients with RA (Lanes 10-13) and from a normal individual (Lane 14), are indicated in the figure and were subsequently analysed by densitometric scanning.

Key:

J  Jurkat standard sample
SLE  Samples from patients with systemic lupus erythematosus
RA  Samples from patients with rheumatoid arthritis
N  Sample from a normal individual
Figure 2.5. Representative autoradiograph of an actin immunoblot. Samples from the Jurkat standard (Lane 1), patients with SLE (Lanes 2-9), patients with RA (Lanes 10-13) and from a normal individual (Lane 14), are indicated in the figure and were subsequently analysed by densitometric scanning.
Methods

corresponding value, using the exact same methods as presented in this dissertation, was recorded in the study of Dhillon, (1993b).
Methods

Section 2.3: Quantitative Determination of Cytokines in SLE

Serum samples from individual patients with SLE or RA and normal individuals, were prepared and stored using the methods outlined in Section 2.2 of this chapter. For details of patients and normal control subjects see Section 2.2, Table 2.2 and Table 2.3. The methods outlined in this section, use the principle of solid phase enzyme-linked immuno-sorbent assays (ELISAs). Alternative laboratory-calibrated methods, which utilise the same ELISA principle, had originally been employed for the measurement of IL-6 and IL-10 in serum samples. However, these were not found to be of satisfactory sensitivity for the determination of levels of IL-6 and IL-10 in serum from normal individuals. Hence, as outlined below, the assays detailed in this section, have been chosen for their high sensitivity in detection of IL-6 and IL-10 within serum.

Quantitative Determination of Interleukin-6

Principle of the assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6, is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. After an incubation period, an amplifier solution is added to the wells and colour develops in proportion to the amount of IL-6 bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

Colour Development

This assay uses an amplification system in which the alkaline phosphatase reaction provides a cofactor that activates a redox cycle leading to the formation of a coloured product. In this system, alkaline phosphatase dephosphorylates the reduced form of nicotinamide adenine dinucleotide phosphate NADPH (Substrate), to reduced nicotinamide adenine dinucleotide (NADH). The NADH subsequently serves as a
specific cofactor that activates a redox cycle driven by the secondary enzyme system, consisting of alcohol dehydrogenase and diaphorase (Amplifier). In the reaction catalysed by diaphorase, NADH reduces a tetrazolium salt (iodonitrotetrazolium violet) to produce a coloured formazan dye and NAD$^+$. NAD$^+$ is in turn reduced by ethanol, in an alcohol dehydrogenase-catalysed reaction, to regenerate NADH which can then re-enter the redox cycle. The rate of reduction of the tetrazolium salt and thus the amount of coloured product formed, are directly proportional to the amount of IL-6 bound in the initial step.

**Experimental Measurements**

This assay contains highly purified E.coli-expressed recombinant human IL-6 and antibodies raised against recombinant human IL-6. The manufacturers have stated that results obtained using natural human IL-6, produced linear curves which parallel the standard curves obtained when using the E.coli-expressed recombinant human IL-6 standards. Thus, these results indicate that this assay can be used to calculate relative mass values for natural IL-6. Moreover, since this assay was insensitive to the addition of recombinant soluble IL-6 receptor (described in Chapter 1, Section 1.1), the manufacturers also stated that experimental IL-6 values are likely to reflect total IL-6 present (free IL-6 and IL-6 bound to soluble receptors). The calibration of experimental IL-6 values (detailed in Appendix B) with International Units, is described later in this section.

**Materials**

Reagents were purchased from R & D Systems Europe, Abingdon, Oxon, U.K. The Quantikine™ HS (high sensitivity) Human IL-6 Immunoassay, consisted of the following reagents.

**IL-6 Microplate**

96 well polystyrene microplate (12 strips of 8 wells) coated with a murine monoclonal antibody against IL-6.

**IL-6 Conjugate**

21 ml of polyclonal antibody against IL-6, conjugated to alkaline phosphatase.
Methods

**Assay Diluent**
6ml of a buffered protein base with preservative.

**Calibrator Diluent**
21ml of animal serum with preservative.

**Wash Buffer Concentrate**
100ml of a five-fold concentrated solution of buffered surfactant.

**Substrate**
Lyophilised NADPH with stabilisers.

**Substrate Diluent**
7ml of buffered solution with stabilisers.

**Amplifier**
Lyophilised amplifier enzymes with stabilisers.

**Amplifier Diluent**
7ml of buffered solution containing ethanol and INT-violet with stabilisers.

**Stop solution**
6ml of 2M sulphuric acid.

Methods

**Summary of the Assay Procedure**

1. All reagents and standards were prepared according to the manufacturers instructions.
2. 50 µl of Assay Diluent was added to each well of the microtiter plate.
3. 200 µl of prepared standard or serum sample was added to each well and incubated at room temperature for 20 hr.
4. Wells were washed four times using the provided Wash Buffer reagent.
5. 200 µl of IL-6 conjugate was added to each well and incubated for 60 mn at room temperature.
6. Wells were washed six times using the provided Wash Buffer reagent.
Methods

7. 50 µl of Substrate Solution was added to each well and incubated for 60 mn at room temperature.
8. 50 µl of Amplifier Solution was added to each well and incubated for 30 mn at room temperature.
9. 50 µl of Stop Solution was added to each well and the absorbance at 490 nm was read within 30 mn and corrected with absorbance measurements at 650 nm.

Calculation of Results

Average readings from each standard and sample were subtracted from the average zero standard optical density. The optical density of the standards versus the concentration of the standards were plotted and a standard curve was obtained for each assay and used to determine the quantity of IL-6 in each patient serum sample. For diluted serum samples, the concentrations read from the standard curve were multiplied by the dilution factor.

Precision

Inter-assay precision was reported by the manufacturers as 0.09–0.81 standard deviations. Five patient serum samples (IL-6 range: 0.2-180 pg/ml) were used in subsequent assays, and the variation of experimental IL-6 values was typically found to be in this reported range.

Calibration of Sample Values

The NIBSC/WHO International Reference Standard 89/548, has been evaluated by the manufacturers, in this assay. The dose response curve of this standard, parallels the curve obtained using the IL-6 standard provided by the manufacturers. The following equation can be used to convert sample values (detailed in Appendix B) using this assay, to the equivalent International Reference Standard value:

NIBSC (89/548) equivalent value (IU/ml) = 0.131 x Quantikine HS IL-6 value (pg/ml)
Methods

Quantitative Determination of Interleukin-10

Principle of the Assay

This assay is a solid phase sandwich ELISA. An antibody specific for human IL-10 has been coated onto the wells of a microtiter plate. Samples, including standards of known human IL-10 content, control and unknown serum specimens, are pipetted into these wells. During the first incubation (see Methods below) the IL-10 antigen binds to the immobilised antibody on the microtiter plate. After washing, a biotinylated antibody specific for human IL-10 is added. During the second incubation, this antibody binds to the immobilised human IL-10 “captured” during the first incubation. After removal of excess secondary antibody, Streptavidin-Peroxidase enzyme is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon to produce colour. The intensity of this coloured product is directly proportional to the concentration of human IL-10 present in the original specimen.

Materials

Reagents were purchased from Biosource International, Inc., California, U.S.A. The Biosource Cytoscreen™ Human IL-10 US (ultra sensitive) immunoassay, consisted of the following reagents.

IL-10 Microplate

96 well polystyrene microplate (12 strips of 8 wells) coated with anti-human IL-10 monoclonal antibodies.

IL-10 Biotin Conjugate

Biotin-labelled anti-human IL-10 antibody (contains 15 mM sodium azide).

Assay Diluent

A buffered protein base with preservative (contains 15 mM sodium azide).
Methods

Wash Buffer Concentrate

100ml of a 25-fold concentrated solution of buffered surfactant

Streptavidin-Peroxidase

Streptavidin-Peroxidase (HRP), 100x concentrate (contains 3 mM thymol)
Streptavidin-Peroxidase (HRP), Diluent (contains 3 mM thymol)

Stabilised Chromogen

Tetramethylbenzidine (TMB)

Stop solution

6ml of 2M sulphuric acid.

Methods

Summary of the Assay Procedure

1. 100 µl of standards and serum samples were added to the microtiter plate wells.
2. Plate was covered and incubated for 3 hr at 37°c.
3. Wells were thoroughly aspirated and washed six times with Wash Buffer.
4. 100 µl of biotinylated anti-IL-10 antibody was added to each well.
5. Plate was covered and incubated for 1 hr at room temperature.
6. Wells were thoroughly aspirated and washed six times with Wash Buffer.
7. 100 µl of Streptavidin-HRP working solution was added to each well.
8. Plate was covered and incubated for 1 hr at room temperature.
9. Wells were thoroughly aspirated and washed six times with Wash Buffer.
10. 100 µl of Stabilised Chromogen was added to each well.
11. Plate was covered and incubated for 35 mn at room temperature.
100 µl of Stop Solution was added to each well and the absorbance at 450 nm was read within 2 hr.
Methods

Calculation of Results

Average readings from each standard and sample were subtracted from the average zero standard optical density. The optical density of the standards versus the concentration of the standards were plotted and a standard curve was obtained for each assay and used to determine the quantity of IL-10 in each patient serum sample. For diluted serum samples, the concentrations read from the standard curve were multiplied by the dilution factor.

Precision

Inter-assay precision was reported by the manufacturers as 0.32-1.72 standard deviations. Five patient serum samples (IL-10 range: 1.8-31.2 pg/ml) were used in subsequent assays, and the inter-assay variation of these experimental IL-10 values was typically found to be in this reported range.
Methods

Section 2.4: ELISA for Anti-Hsp Antibodies

This assay utilises the protocol of Conroy et al., (1994; 1996) for the detection of antibodies to Hsp 90 or Hsp 70 within serum samples obtained from patients with SLE or RA and normal individuals.

Principle of the Assay

This assay is a solid phase sandwich ELISA. Purified Hsp 90 or Hsp 70 is coated onto the wells of a microtiter plate. Samples, including standards (antibodies to human Hsp 90 or Hsp 70), are pipetted into these wells. During the first incubation (see Methods below) serum anti-Hsp antibodies bind to the immobilised Hsp antigen on the microtiter plate. After washing, an alkaline phosphatase-conjugated antibody specific for human IgG or IgM is added. During the second incubation, this antibody binds to the immobilised human anti-Hsp antibody “captured” during the first incubation. After removal of excess secondary antibody, and washing to remove all the unbound moieties, a substrate solution is added, which is acted upon to produce colour. The intensity of this coloured product is directly proportional to the concentration of human anti-Hsp antibody present in the original specimen.

Materials

Monoclonal Antibody Standards

Monoclonal antibody AC16 was a gift from Professor D.O. Toft, Mayo Clinic Foundation, Rochester, Minnesota, USA. Antibody AC16 is raised in rats and is of class IgGj and has been demonstrated to react with human Hsp 90 and binds to the native form with greater affinity than to denatured human Hsp 90 (private communication). Mouse monoclonal antibody N27F3-4 (class IgGj) was purchased from Stressgen Biotechnologies Inc., Canada and reacts with human Hsp 73 and with faint reactivity to human Hsp 72. This was confirmed in the study of Dhillon (1993b).
Methods

Preparation of Purified Hsp 90 and Hsp 70

Purified Hsp 90 or Hsp 70 (Stressgen, Canada) was reconstituted in sterile phosphate buffered saline to a concentration of 1 mg/ml as single-use aliquots and stored at -70°C until required. Prior to use, frozen protein samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), according to the method of Laemmli, (1970) and Western blotting, using the methods outlined in Section 2.1 of this chapter, to check there was only one discrete band.

Methods

ELISA for Anti-Hsp 90 Antibodies

Nunc ELISA (Dynatech) plates were coated with 100 µl per well of purified Hsp 90 (Stressgen, Canada) at 0.5 µg/ml in 0.05 M bicarbonate buffer (pH 9.6), overnight at 4°C. Half of each plate was coated only with 0.05 M bicarbonate buffer. Sera were diluted 1:200 in PBS supplemented with 0.1% v/v polyoxyethylene sorbitan monolaurate (Tween20), 1% (v/v) goat serum (Sigma, U.K) and 1% w/v BSA. 100 µl of diluted sera were added to the ELISA plate in duplicate wells and incubated at 37°C for 1 hr. After five washes with PBS-Tween, 100 µl of anti-human IgG or IgM F(ab')2 alkaline phosphatase-conjugated antibodies, was added to wells at a dilution of 1:10,000 and 1:50,000 respectively, in PBS supplemented with 0.1% v/v polyoxyethane sorbitan monolaurate (Tween20), 1% goat serum (Sigma, U.K) and 1% w/v BSA. This was incubated at 37°C for 1 hr, followed by five washes with PBS-Tween and then two washes with bicarbonate buffer.

Colour was developed using p-nitrophenyl phosphate tablets (Sigma, U.K) as substrate. Two tablets (each 5 mg) were dissolved in 10 ml of bicarbonate buffer to which 20 µl of 1 M MgCl₂ had been added. The reaction was allowed to develop at 37°C for 30 min. Plates were read on a Dynatech 4000 ELISA reader (Dynatech, Billinghurst, UK) at 405 nm and corrected at 490 nm.
Methods

ELISA for Anti-Hsp 70 Antibodies

Nunc ELISA (Dynatech) plates were coated with 100 μl per well of purified Hsp 70 (Stressgen, Canada) at 1.0 μg/ml in 0.05 M bicarbonate buffer (pH 9.6), overnight at 4°C. Half of each plate was coated only with 0.05 M bicarbonate buffer. Sera were diluted 1:500 in PBS supplemented with 0.1% v/v polyoxyethylene sorbitan monolaurate (Tween20), 1% (v/v) goat serum (Sigma, U.K) and 1% w/v BSA. 100 μl of diluted sera were added to the ELISA plate in duplicate wells and incubated at 37°C for 1 hr. After five washes with PBS-Tween, 100 μl of anti-human IgG or IgM F(ab')2 alkaline phosphatase-conjugated antibodies were added to wells at a dilution of 1:10,000 and 1:50,000 respectively, in PBS supplemented with 0.1% v/v polyoxyethylene sorbitan monolaurate (Tween20), 1% goat serum (Sigma, U.K) and 1% w/v BSA. This was incubated at 37°C for 1 hr, followed by five washes with PBS-Tween and then two washes with bicarbonate buffer.

Colour was developed using p-nitrophenyl phosphate tablets (Sigma, U.K) as substrate. Two tablets (each 5 mg) were dissolved in 10 ml of bicarbonate buffer to which 20 μl of 1 M MgCl₂ had been added. The reaction was allowed to develop at 37°C for 2 hr. Plates were read on a Dynatech 4000 ELISA reader (Dynatech, Billinghurst, UK) at 405 nm and corrected at 490 nm.

Calculation of Results

Average readings from each sample were subtracted from the average OD values obtained from the wells with no antigen and was expressed as a percentage of a positive control sera (containing AC16 to Hsp 90 or antibody N27F3-4 to Hsp 70) applied in duplicate on each plate. A known negative serum was also applied in duplicate to each plate. Patients were considered to have a raised level of anti-Hsp antibody if the corrected OD value was greater than the 97.5th centile of 30 healthy normal controls.
Methods

Section 2.5: Disease Associations

In the following results chapters, levels of Hsps, cytokines and antibodies to Hsps have been determined in individual blood samples from patients with SLE, using the methods outlined in this chapter. Subsequently, these data have been compared with clinical disease activity in individual patients, recorded at the time of providing a sample of blood.

In a previous study (Dhillon et al., 1993a) levels of Hsps determined in individual blood samples from patients with SLE, were analysed using the UCH/Middlesex SLE disease activity scoring system (Morrow et al., 1982; 1983). Thus, using this index, disease activity in patients was classified as inactive, mildly active, moderately active or severely active. However, the yield of information using this index is relatively low. For example, disease which is scored as overall mildly active, may be masking severe disease activity in one particular organ (Dhillon, 1993b). For this reason, the study of Dhillon et al., (1993a) also used the The British Isles Lupus Assessment Group (BILAG) index for scoring clinical disease activity in patients with SLE.

The BILAG index is a standardised measure of clinical disease activity in SLE, which has been validated internationally against other methods of scoring SLE activity, including the UCH/Middlesex scoring system (Symmons et al., 1988), (Isenberg, 1990), (Gladman et al., 1992). The BILAG index (Symons et al., 1988) defines disease activity within eight organs or systems: general disease, mucocutaneous disease, musculoskeletal disease, cardiovascular disease and respiratory disease, vasculitis, renal disease and haematological disease (see Chapter 1, Section 1.3 and Appendix A: The BILAG Index).

An alphabetic score (A-E) is determined for each category on the basis of the physician’s intention to treat the patient. Score “A” corresponds to severely active disease requiring major immunosuppressive therapy or a substantial increase in current therapy; a score “B” corresponds to active disease without substantial alteration to current therapy; a score “C” corresponds to mild disease or evidence of previous disease; a score “D” corresponds to no current disease or evidence of previous disease; and a score “E” corresponds to no current/never any disease in a category. BILAG global scores in a patient with SLE are determined by the summation of BILAG scores in each organ/system, where a score “A”= 9 points, a score “B”= 3 points, a score “C”= 1 point and a score “D” or “E”= 0 points.
Methods

Patients attending the morning outpatient SLE Clinic at the Centre for Rheumatology, Department of Medicine, University College London, are routinely assessed for clinical disease activity, using the BILAG index. Thus, detailed records of these data have been recorded for 90% of the patients with SLE studied in this dissertation. Thus, in Chapter 6 of this dissertation, levels of Hsps, cytokines and antibodies to Hsps have been compared in patients with SLE graded by the BILAG index. For the purpose of statistical analyses (see Section 2.5 of this chapter), a patient with a score “A-C” recorded in a category, was considered as having “active disease” in that category; a score “D” or “E” was considered as “inactive disease” in that category.
Methods

Section 2.6: Statistical Analyses

Statistical analyses were performed using an SPSS computer software package (SPSS Science, Chicago, U.S.A) and with helpful discussions and advice from senior lecturers of the Department of Medical Statistics, Institute of Child Health, University College London. Information on the various statistical tests used in the following results chapters, is detailed in this section and has been obtained from Altman, (1999).

Summary statistics, of data for levels of Hsps (Table 4.2.1) and cytokines (Table 4.3.1) in all subject groups are provided in Chapter 4 and of data for levels of antibodies to Hsps (Table 5.2.1) in Chapter 5. Following advice from the Department of Medical Statistics, levels of the cytokines IL-6 and IL-10 were log_{10}-transformed, to reduce any influence of outlying and thus atypical values present in the raw data on subsequent statistical analyses. As described below, parametric and non-parametric methods of statistical analysis were performed on all these data.

Differences between mean levels of Hsps or between mean levels of cytokines determined for two independent subject groups (see Chapter 4 and Chapter 6), were assessed for statistical significance using the student's t-test. The use of the two sample t-test is based on the assumption that the variances of the two populations are the same. However, the t-test is known to be robust in that it is little affected by moderate failure to meet this assumption. The null hypothesis that the data from each group have equal variances is assessed by the SPSS statistics package, using the variance ratio test (F-test). The variance ratio is calculated as the square of the ratio of the sample standard deviations. The distribution of the F statistic has two values of degrees of freedom, one corresponding to each variance in the data for each group. Where variances are determined to be unequal, a modified student's t-test is applied to the data. Analyses involving student's t-tests are presented as bar charts with error bars representing 95% confidence intervals (95% CI: -2SEM to +2SEM) for the mean of a variable in a subject group. P values denote the significance of the difference in mean values of a variable between two independent groups. A P value of less than 0.05 (P<0.05), was considered to be significant.

Analyses of the correlation between levels of Hsps and cytokines, in individual samples from patients with SLE, are presented in Chapter 4. Correlation is a method of analysis for studying the possible association between two variables. The degree of association is measured by calculating the correlation coefficient. The standard method (Pearson) leads to a quantity called "r" which measures the degree of "straight-line" association between the values of the variables and can take any value from -1 to +1.
Methods

Thus, a value of +1 or -1 is obtained, if all data points in a scattergraph of the type shown in Chapter 4, lie in a straight line. Scattergraphs in Chapter 4 showing an associated trend-line, also indicate the value for the Pearson correlation coefficient “r” and the associated probability (P) value. Any value for “r” which generated a P value of less than 0.05 (p<0.05), was considered to be significant. All significant correlations by this criterion presented in Chapter 4, have also attained statistical significance (p<0.05) non-parametrically, using a Spearman’s rank correlation test, which is essentially a Pearson correlation on ranked data. Correlations of BILAG global scores in individual patients with SLE with either levels of Hsps or levels of cytokines determined in individual samples from the same patients (see Chapter 6), were performed non-parametrically, using either a Spearman’s or Kendall’s rank correlation analysis. These methods of correlation were chosen, since the value of a BILAG global score in an individual patient with SLE, is obtained using a highly skewed scoring system (see Section 2.4 of this chapter).

Chi-Square analyses were performed for the evaluation of possible associations between categorical data. For example, in Chapter 4, patients with SLE have been categorised as having normal or elevated levels of Hsp 90, in Chapter 5, as having normal or raised levels of antibodies to Hsp 90 and in Chapter 6, as having active or inactive disease in an organ/system. For all chi-square analyses detailed in these chapters, patients with SLE have been categorised into mutually exclusive groups in relation to two classifications. The null hypothesis of this test, is that the two classifications, for example HLA status and raised anti-Hsp 70 antibody level status, are unrelated in the relevant population. Where a chi-square analysis has been performed, a Pearson P value is provided, which if less than 0.05 (P<0.05), was considered to be significant. Included with all chi-square analyses which have generated a significant Pearson’s P value, are alternative analyses of the categorical data which each have an identical null hypothesis to the chi-square analysis. These include a Fischer’s exact test, a likelihood ratio test and a linear by linear association test (see Altman, 1999). In all cases where a significant Pearson P value has been attained and presented, these tests have also generated significant P values, in agreement with the chi-square analysis.

In principal, interpretations of P values generated by any of the analyses detailed above, can lead to a rejection of a null hypothesis, when in reality it is true (a Type I error), or the acceptance of a null hypothesis, when in reality it is false (a Type II error). In all analyses detailed above, the probability of obtaining a Type I error has been predetermined as 5%. Thus, by this criterion, a P value of less than 0.05 (P<0.05) has been considered significant. In some cases however, multiple student’s t-tests and chi-square tests have been performed, involving the same variable (continuous or categorical). For
example, in Chapter 4, six individual t-tests have been performed, to compare mean levels of Hsp 90 in patients with SLE, with respect to the presence or absence of individual/combined HLA alleles/haplotypes. Multiple analyses of this type, are likely to lead to Type I errors just by chance. There is no simple or totally satisfactory solution to this problem. Several methods have been proposed to deal with this problem, such as Bonferroni, which adjusts P values obtained from multiple student’s t-tests or chi-square analyses, such that for \( k \) paired comparisons, P is adjusted to \( kP \). However, methods of this type may be reasonable for up to five numbers of comparisons, but for large numbers its use is highly conservative. If research aims justify large numbers of comparisons, then an alternative approach is to quote exact P values and to reduce the cut-off level for statistical significance (Altman, 1999 and advice from the Department of Medical Statistics). Thus, by this criterion, P values obtained in multiple analyses presented in the following chapters, have been considered significant if the value of P is less than 0.02 (P<0.02). Where the results of analyses of this type are in agreement with previous studies, no adjustment to the 5% cut-off level for significance has been made.
Results

Chapter 3

The Effect Of Interleukin-10 On Hsp 90
Gene Expression

3.0 Introduction

Elevated circulating levels of the cytokine interleukin-10 (IL-10) have been reported in patients with SLE and are typically highest in patients with active disease (Park et al., 1998). In view of the function of IL-10 within the normal immune system, a pathogenic role for IL-10 in SLE has been suggested and is supported by a number of studies, which are discussed in Chapter 1, Section 1.3. Any role for IL-10 in the pathogenesis of SLE is likely to involve the induction of the expression of specific genes within its target cells, resulting in changes of cellular phenotype.

The 90 kDa heat shock protein (Hsp 90) is elevated in specific subsets of patients with SLE, primarily in patients with active central nervous system and cardiovascular/respiratory disease (Dhillon et al., 1993a; 1994), see Chapter 1, Section 1.4. Further studies have shown that this elevation is dependent on enhanced transcription of the Hsp 90β gene (Twomey et al., 1993). Although the exposure of cells to elevated temperatures and other stresses results in the induction of a number of different Hsps including Hsp 90 (see Chapter 1, Section 1.1), the elevation of Hsp 90 observed in SLE is a specific effect on Hsps and is not paralleled by elevation of the constitutively expressed heat shock proteins Hsp 73 or Hsp 60. A role for Hsp 90 both in the aetiology and pathogenesis of SLE has been discussed in Chapter 1, Section 1.4, however, the precise mechanisms leading to the overexpression of Hsp 90 have remained unclear.
Recent studies (outlined in Chapter 1, Section 1.1) have shown that the cytokine interleukin-6 (IL-6), which is also present at elevated levels in patients with active SLE (Linker-Israeli et al., 1991), up-regulates Hsp 90 expression in the human hepatoma cell line HepG2 and in peripheral blood mononuclear cells (PBMCs) from normal individuals (Stephanou et al., 1997). An investigation into the mechanisms of Hsp 90 promoter activation in response to IL-6 has been recently reported (Stephanou et al., 1998b). In this study, using reporter gene assays, activation of the Hsp 90 promoter by IL-6 is mediated by a region of the promoter (from -643 to -623 relative to the transcriptional start site) which contains binding sites for the IL-6 activated transcription factors STAT-3 and NF-IL6. It is interesting to note that IL-10 and IL-6 utilise similar signalling mechanisms for modulating gene expression within their target cells. As discussed in Chapter 1, Section 1.1, IL-10 has been shown to mediate intracellular signalling via the JAK/STAT pathway, in which stimulation of receptor-associated Jaks (Jak1 and Tyk2) leads to phosphorylation and activation of the transcription factors STAT-1 and STAT-3 (Finbloom and Winestock, 1995).

The similarity of gene regulatory elements targeted by signalling pathways for both IL-6 and IL-10 through the activation of STATs, together with studies supporting a role for IL-10 in the pathogenesis of SLE (see Chapter 1, Section 1.3), has therefore prompted an investigation of the effect of IL-10 on Hsp 90 gene expression.
Results

3.1 IL-10 Upregulates Hsp 90 in HepG2 Cells and PBMCs

The effect of IL-10 on Hsp 90 gene expression was investigated by treatment of both HepG2 cells and PBMCs with IL-10 and levels of Hsp 90 measured by Western blot analysis (Figure 3.1). IL-10 was clearly observed to up-regulate Hsp 90 levels within the HepG2 cell line (Figure 3.1a) which had been artificially engineered to express the human IL-10 receptor. Within the parental cell line (lacking the IL-10 receptor) no elevation of Hsp 90 was observed following treatment with IL-10 (result not shown). The 30 kDa band in Figure 3.1 results from the cross-reactivity of antibody AC88 with heterogenous ribonuclear proteins, as detailed in Chapter 2. In cells cotransfected with an expression vector for STAT-3 and then stimulated with IL-10, Hsp 90 levels were also observed to be elevated. Elevated levels of Hsp 90 were also observed in unstimulated cells transfected with STAT-3 compared to control unstimulated cells.

In PBMCs, Hsp 90 levels are significantly elevated following IL-10 treatment, compared to control untreated cells (Figure 3.1b). This effect is also observed in PBMCs treated with interferon-γ and IL-6, which has been reported previously (Stephanou et al., 1998b). Furthermore, an analysis of Hsp 90 levels in PBMCs following a time-course of IL-10 treatment (Figure 3.1c) shows that elevation of Hsp 90 levels is rapid. An approximate three-fold increase in the level of Hsp 90 is observed after 1 hr of IL-10 treatment compared to control untreated cells, with this increase being sustained for 48 hr of treatment.
Figure 3.1. IL-10 Up-Regulates Hsp 90 in HepG2 Cells and PBMCs

(a) Western blot with antibody AC88 to Hsp 90. HepG2 cells were treated with either 50 ng/ml (50) or 100 ng/ml (100) of IL-10 for 12 hr or untreated (0). Cells transfected with 2 μg of a STAT-3 expression vector are shown (+3).

(b) Western blot with antibody AC88 to Hsp 90. PBMCs were untreated (lanes 1 and 2), treated with interferon γ (lane 3), IL-6 (lane 4), or treated for 16 hr with 50 ng/ml of either IL-6 (lane 5) or IL-10 (lane 6).
Figure 3.1c. Time course of Hsp 90 elevation in PBMCs treated with IL-10. PBMCs were untreated or treated with 100 ng/ml of IL-10 for the times indicated in the figure. Values for Hsp 90 levels represent fold induction relative to control untreated cells.
Results

3.2 IL-10 Activates the Hsp 90α and -β Gene Promoters

To investigate the mechanisms by which IL-10 induces enhanced Hsp 90 levels in HepG2 cells, the effect on the Hsp 90 promoter was studied using plasmid constructs in which the Hsp 90α and -β promoters are coupled to a CAT reporter gene. As shown in Figure 3.2a and Table 3.1, IL-10 significantly activates both the Hsp 90α and -β promoters, as measured by CAT activity in IL-10-treated and untreated cells. Cells cotransfected with a STAT-3 expression vector and treated with IL-10, exhibit a further slight increase in CAT activity compared to IL-10-treated cells. In view of the known effects of IL-6 on Hsp 90 gene regulation, IL-6 treatment of cells transfected with Hsp 90 promoter constructs, was used to compare the responsiveness of the reporter constructs to IL-10 and IL-6. Figure 3.2b illustrates the responsiveness of the Hsp 90β promoter construct to both IL-6 and IL-10 and indicates that the two effects are comparable.

3.3 IL-10 Activates the Hsp 90β Promoter via a STAT-3 Binding Site

In view of previous studies which have shown that a region of the Hsp 90β promoter (-643 to -623 relative to the transcriptional start site) can confer responsiveness to IL-6 when linked to a heterologous promoter, the responsiveness of this region to IL-10 was investigated. HepG2 cells were transfected with construct Hsp 90-S3E, corresponding to this region linked to the heterologous thymidine kinase promoter, or with construct Hsp 90-MS3E, in which the wild-type STAT-3 site has been mutated (see Chapter 2, Figure 2.1). CAT activity of the constructs was then measured following treatment of cells with IL-10. Figure 3.3a and Table 3.1 show that the wild-type STAT-3 sequence gave high levels of CAT activity in response to IL-10 and activation was enhanced by cotransfection with a STAT-3 expression vector. Conversely the mutant construct (Figure 3.3b and Table 3.1) was not responsive to IL-10 and no significant increase in CAT activity was observed following cotransfection with STAT-3.
Figure 3.2 Assay of CAT Activity in HepG2 Cells Transfected with Hsp 90α and -β Promoter-CAT Constructs

(a) 1 2 3 4 5 6

(b) I – 10 6 10 6 –
   S – – – + + +
Results

Legend to Figure 3.2

(a) HepG2 cells were transfected with 2 μg of either Hsp 90α-CAT (lanes 1-3) or Hsp 90β-CAT (lanes 4-6). Cells were left untreated (lanes 1,4) or treated with 100 ng/ml IL-10 for 12 hr (lanes 2,3 and 5,6). Lanes 3 and 6 correspond to cells cotransfected with 1 μg of a STAT-3 expression vector.

(b) Assay of CAT activity in HepG2 cells transfected with Hsp 90β-CAT and treated with IL-6 or IL-10. HepG2 cells were transfected with 2 μg of Hsp 90β-CAT and treated (I) with either 50 ng/ml IL-6 (6) or 100 ng/ml IL-10 (10) for 12 hr or untreated (−). Cells cotransfected (S) with 1 μg of a STAT-3 expression vector are denoted (+).
Figure 3.3  IL-10 Activates the Hsp 90β Promoter Via a STAT-3 Binding Site

(a)  

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>10</th>
<th>10</th>
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<td>S</td>
<td>-</td>
<td>-</td>
<td>+</td>
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(b)  

<table>
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<tr>
<th></th>
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<td>S</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
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</table>
Results

Legend to Figure 3.3

Assay of CAT activity in HepG2 cells transfected with Hsp 90-S3E (a) or Hsp 90 MS3E (b). HepG2 cells were transfected with 2 μg of either CAT reporter construct and treated (I) with 100 ng/ml IL-10 (10) for 12 hr or untreated (–). Cells cotransfected (S) with 1 μg of a STAT-3 expression vector are denoted (+).
Table 3.1  Effect of IL-10 on the Activity of Hsp 90 Promoter CAT Constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>- IL-10</th>
<th>+ IL-10</th>
<th>+IL-10 + STAT-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp 90α-CAT</td>
<td>4.6</td>
<td>61.8</td>
<td>88.0</td>
</tr>
<tr>
<td>Hsp 90β-CAT</td>
<td>2.5</td>
<td>78.2</td>
<td>84.2</td>
</tr>
<tr>
<td>pBLCat2 vector</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Hsp 90-S3E</td>
<td>1.3</td>
<td>46.0</td>
<td>48.1</td>
</tr>
<tr>
<td>Hsp 90-MS3E</td>
<td>2.9</td>
<td>4.0</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Values represent the CAT activity of each construct either alone or cotransfected with a STAT-3 expression vector (+ STAT-3) in HepG2 cells treated (+ IL-10) or untreated (-IL-10) with IL-10 for 12 hr before harvesting. Values were obtained by scanning densitometry of assays of the type shown in Figs 2,3,4.
Results

3.4 Discussion

Although the regulation of heat shock proteins by stressful stimuli has been intensively studied, much less attention has been given to their regulation by non-stressful stimuli, such as cytokines. This is of particular importance, since Hsp expression has been observed to be regulated during a number of normal cellular events such as T-cell activation (Haire et al., 1988) and the differentiation of monocytes to macrophages (Twomey et al., 1993). Hence, such regulatory mechanisms may play a key role in determining the level of Hsp expression in unstressed cells, as well as being important in their overexpression in particular disease states.

It has previously been demonstrated that treatment of cells with IL-6 can up-regulate Hsp 90 levels in vitro (Stephanou et al., 1997) and that such elevation of Hsp 90 levels is also observed in vivo in transgenic mice overexpressing IL-6 (Stephanou et al., 1998a). This effect is due to the IL-6-induced activation of the transcription factors NF-IL6 and STAT-3 and is mediated via a short specific region of the Hsp 90 promoter which contains binding sites for these factors (Stephanou et al., 1997; 1998b). In this chapter, these observations have been extended by showing that IL-10 can also enhance Hsp 90 levels, activating the Hsp 90β gene promoter via the same short element that is targeted by IL-6. As IL-10 activates STAT-3 but not NF-IL6 (Finbloom and Winestock, 1995), it is likely that this effect is mediated via the IL-10-induced activation of STAT-3 and in agreement with this, the ability of IL-10 to activate the Hsp 90α and -β gene promoters is enhanced by cotransfection of a STAT-3 expression vector. Hence the activation of Hsp 90 by IL-10 is likely to reflect the similarity of its signalling pathway to that of IL-6 and indicates that such activation can occur in the absence of activation of NF-IL6 which is achieved only by IL-6. This is in accordance with the observation that enhanced Hsp 90 levels are observed both in transgenic mice containing the IL-6 gene and in transgenic mice in which the gene encoding NF-IL6 has been inactivated, resulting in enhanced levels of IL-6 (Stephanou et al., 1998a).
Results

The induction of Hsp 90 expression by both IL-6 and IL-10 infers a role for Hsp 90 expression both within the normal functioning of the immune system and as part of an immune response. It is likely that both IL-6 and IL-10 regulate the expression of Hsp 90, ensuring appropriate levels of chaperoning functions of Hsp 90 within immune cells. More importantly, these results have important implications for our understanding of the mechanisms of Hsp 90 regulation in SLE. Thus, since, IL-6 (Linker-Israeli et al., 1991) and IL-10 (Park et al., 1998), (Linker-Israeli, 1992) are elevated in SLE, whilst studies suggest a role for Hsp 90 in the pathogenesis of autoimmunity in this disease (Stephanou et al., 1998c), an investigation into the relative effects of these two factors in producing the Hsp 90 elevation observed in SLE, was carried out and is discussed in the following results chapter.
Chapter 4

Cytokines and Heat Shock Protein
Gene Expression in SLE

4.0 Introduction

As described in detail in Chapter 1 (Section 1.3), systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease mainly confined to women during the child-bearing years, that is found in all ethnic groups but is particularly common amongst Afro-Carribbeans (Johnson et al., 1995). SLE is characterised by B lymphocyte hyperactivity and decreased cellular immune responses. Such immune disturbances may in part reflect abnormalities in the expression of cytokines which regulate the functions of cells within the normal immune system. In agreement with this, elevated levels of the cytokines interleukin-6 (Linker-Israeli et al., 1991) and interleukin-10 (Park et al., 1998) have been reported in patients with SLE and are highest in patients with active disease. Any role for cytokines in the pathogenesis of SLE, must involve the activation of specific genes in target cells. Understandably, the identification of these genes and dissection of the molecular mechanisms by which they are regulated both in vitro and in vivo, is of importance.

The heat shock proteins (Hsps) are a group of proteins whose synthesis is enhanced in cells in response to a variety of stressful and non-stressful conditions (see Chapter 1, Section 1.1). Elevated levels of Hsp 90 have been reported in up to 30% of patients with SLE (Dhillon et al., 1993a). Intriguingly, this overexpression is found most often in patients who are not HLA A1, B8, DR3, the most common haplotype in caucasians who have the disease (Dhillon et al., 1994). Evidence of a potential role for Hsp 90 in the aetiology and pathogenesis of this disease has been
Results

supported by studies in autoimmune MRL-lpr/lpr mice where elevated levels of Hsp 90 are detectable prior to the onset of disease (Faulds et al., 1994).

The precise mechanisms leading to the observed elevation of Hsp 90 in patients with SLE, have however remained unclear. Previous studies which have suggested a role for the cytokine interleukin-6 (IL-6) in the regulation of Hsp 90 in SLE (see Chapter 1, Section 1.1), have been extended in Chapter 3 to show that in vitro, the cytokine interleukin-10 (IL-10) up-regulates Hsp 90 in a human hepatoma cell line (HepG2) and in peripheral blood mononuclear cells (PBMCs) from normal individuals (Ripley et al., 1999). Thus it is conceivable that both IL-6 and IL-10 are involved in the regulation of Hsp 90 in vivo, within cells of the normal immune system. In addition, the elevated circulating levels of IL-6 (Linker-Israeli et al., 1991) and IL-10 (Park et al., 1998) which have been reported in patients with SLE, would suggest a role for both cytokines in the overexpression of Hsp 90 in this disease.

In this chapter, an investigation of the correlation between IL-6 or IL-10 levels and the levels of heat shock proteins in PBMCs, from normal individuals and patients with SLE, is outlined. In view of previous studies which suggested a genetic association with the overexpression of Hsp 90 in SLE (Dhillon et al., 1994), it was thought to be of interest to investigate in parallel, any effect of HLA status on the involvement of cytokines in the regulation of Hsps in patients with SLE.
4.1 Quantitative Determination of Heat Shock Proteins

Levels of Hsp 90 and Hsp 70 in peripheral blood mononuclear cells (PBMCs) from patients with SLE and controls (normal individuals and patients with rheumatoid arthritis) were determined by the relevant methods outlined in Chapter 2.

4.2 Pattern of Heat Shock Protein Expression in SLE

Table 4.2.1 shows the mean, 95% confidence interval of the mean (-2SEM to +2SEM), median and range, of the levels of Hsp 90 and Hsp70 in normal individuals and patients with SLE or rheumatoid arthritis (RA). The mean level of Hsp 90 in patients with SLE was found to be significantly higher (p<0.01) than the mean Hsp 90 level determined for the normal control group. In contrast, the mean Hsp 90 level in patients with RA was not observed to be significantly higher than the mean Hsp 90 level of the normal control group. No significant differences in the mean Hsp 70 level were observed in patients with either SLE or RA, compared to the normal control group. Thus, the observed pattern of Hsp levels in patients with SLE or RA, compared to normal individuals, is in agreement with previous studies (Dhillon et al., 1993a) and is discussed in Chapter 1, Section 1.4. Scatter graphs representing the levels of Hsp 90 and Hsp 70 in normal individuals and patients with SLE or RA, are shown in Figure 4.2.1 and Figure 4.2.2.

Table 4.2.2 shows the upper limit of the reference range for each Hsp in the normal control group. The 97.5th centile was used as the upper limit of "normal" for both Hsp 90 and Hsp 70. Table 4.2.2 also shows the number and percentage of patients with SLE or RA, with levels of Hsps above the normal 97.5th centile. Thus, by this criterion, elevated levels of Hsp 90 were observed in 30 patients with SLE (30.61%) and one patient with RA (5%).
Results

Table 4.2.1 Hsp levels in PBMCs of Controls and Patients with SLE

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hsp 90</td>
<td>Hsp 70</td>
</tr>
<tr>
<td>n</td>
<td>95</td>
<td>107</td>
</tr>
<tr>
<td>Mean</td>
<td>2.46</td>
<td>1.81</td>
</tr>
<tr>
<td>95% CIM</td>
<td>1.84 - 3.08</td>
<td>1.67 - 1.93</td>
</tr>
<tr>
<td>Median</td>
<td>1.82</td>
<td>1.73</td>
</tr>
<tr>
<td>Range</td>
<td>15.6</td>
<td>2.32</td>
</tr>
<tr>
<td>IQR</td>
<td>2.68</td>
<td>2.14</td>
</tr>
<tr>
<td>P value</td>
<td>&gt;0.1</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>SE difference</td>
<td>0.36</td>
<td>0.14</td>
</tr>
</tbody>
</table>
### Results

Table 4.2.1 (continued)

<table>
<thead>
<tr>
<th></th>
<th>Hsp 90</th>
<th>Hsp70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid Arthritis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean</td>
<td>1.83</td>
<td>0.67</td>
</tr>
<tr>
<td>95% CIM</td>
<td>1.62-2.04</td>
<td>0.55-0.79</td>
</tr>
<tr>
<td>Median</td>
<td>1.94</td>
<td>0.52</td>
</tr>
<tr>
<td>Range</td>
<td>3.68</td>
<td>2.13</td>
</tr>
<tr>
<td>IQR</td>
<td>1.83</td>
<td>1.12</td>
</tr>
<tr>
<td>P value[^]</td>
<td>&gt;0.6</td>
<td>&gt;0.4</td>
</tr>
<tr>
<td>SE difference</td>
<td>0.08</td>
<td>0.19</td>
</tr>
</tbody>
</table>

**Key:**

- **n**: Number of subjects.
- **CIM**: Confidence interval for mean.
- **IQR**: Value for the interquartile range.
- **P value[^]**: Level of significance for the difference in mean Hsp levels between patients with SLE and normals (P value[^1]) and between patients with RA and normals (P value[^2]), using Student's t-test on these data.
- **SE**: Value for the standard error in the mean Hsp level.
### Results

**Table 4.2.2  Upper Limits of Normal Hsp Levels and Number of Patients with SLE or RA with Elevated Hsp Levels**

<table>
<thead>
<tr>
<th></th>
<th>Hsp 90</th>
<th>Hsp 70</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean + 2SD</td>
<td>3.13</td>
<td>1.45</td>
</tr>
<tr>
<td>97.5(^{th}) centile</td>
<td>2.65</td>
<td>1.32</td>
</tr>
</tbody>
</table>

|                |        |        |
| **SLE**        |        |        |
| Number of patients with elevated Hsp levels (above the normal 97.5\(^{th}\) centile) | 30     | 7      |
| % of patients with elevation   | 30.61  | 6.54   |

|                |        |        |
| **Rheumatoid Arthritis**   |        |        |
| Number of patients with elevated Hsp levels (above the 97.5\(^{th}\) centile) | 1      | 1      |
| % of patients with elevation | 5.00   | 5.00   |
Figure 4.2.1  Hsp 90 levels in SLE and Control Groups

Hsp 90

P< 0.02

<table>
<thead>
<tr>
<th>Group</th>
<th>95</th>
<th>SLE</th>
<th>30</th>
<th>Normals</th>
<th>20</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.2.2  Hsp 70 Levels in SLE and Control Groups

Hsp 70

<table>
<thead>
<tr>
<th>n</th>
<th>Group</th>
<th>107</th>
<th>SLE</th>
<th>30</th>
<th>Normals</th>
<th>20</th>
<th>RA</th>
</tr>
</thead>
</table>
Results

Legend to Figures 4.2.1 and 4.2.2

Scatter graphs of Hsp levels for all groups. Dotted lines show median values and solid lines indicate the 97.5\textsuperscript{th} centile of the Hsp levels in the normal control group.

(p< 0.02) – level of significance for the difference in mean values of Hsp 90 levels between SLE and normal groups, using a Student’s t-test for equality of means. No significant differences in mean Hsp 90 levels were found between RA and normal groups, or for Hsp 72 between any groups.

Key to Abbreviations:

SLE  systemic lupus erythematosus group
C    normal control group
RA   rheumatoid arthritis group
Results

Figure 4.2.3 is a scatter graph comparing levels of Hsp 90 with levels of Hsp 70 in individual samples from patients with SLE. No significant correlation between the levels of Hsp 90 and Hsp 70 in patients with SLE was observed. Thus, the observed elevation of Hsp 90 levels in patients with SLE, compared to normals, was specific to this Hsp, and was not paralleled by an elevation of Hsp 70 levels in patients with this disease compared to normals.

In view of previous studies which have suggested a genetic origin to the elevation of Hsp 90 in SLE (Dhillon et al., 1994), mean levels of Hsp 90 were determined in patients with SLE with respect to the HLA alleles A1, B8 and DR3, which have been previously reported to be strongly associated with SLE (Worrall et al., 1990). Figure 4.2.4 shows mean Hsp 90 levels and 95% confidence limits (-2 SEM to +2 SEM), in patients with SLE with respect to the presence or absence of individual/combined HLA alleles/haplotypes. Significantly higher mean Hsp 90 levels were observed in patients who were HLA DR3-negative compared to those patients who were HLA DR3-positive (P< 0.01) and this was observed for all combined haplotypes in which DR3-negativity was present. Patients lacking the HLA B8 allele were also found to have higher mean Hsp 90 levels compared with those patients who possessed this allele (P<0.05).

In contrast, Figure 4.2.5 shows that no significant differences in mean Hsp 70 levels were associated with either the presence or absence of any of these HLA alleles. A further statistical analysis of the association of A1, B8 and DR3 HLA alleles to Hsp 90 levels in patients with SLE, was applied to the patients with elevated Hsp 90 levels. Table 4.2.3 shows chi-square analyses and levels of significance for this data and highlights HLA DR3 status as being significantly associated with a patient with SLE having elevated levels of Hsp 90. Table 4.2.4 shows the number of patients with elevated or normal levels of Hsp 90, with respect to the presence or absence of the HLA DR3 allele and also details a chi-square analysis of these data (see statistical analyses in Chapter 2).
Figure 4.2.3  Correlation Between Hsp 90 and Hsp 70 Levels in SLE

Scatter graph showing the correlation between levels of Hsp 90 and Hsp 70 determined for individual patients with SLE.
Figure 4.2.4  Hsp 90 mean (and 95% CI of mean) with respect to HLA alleles/haplotype

<table>
<thead>
<tr>
<th>Allele or Haplotype</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>m</td>
<td>9 84</td>
<td>7 87</td>
</tr>
<tr>
<td>A1B8</td>
<td>13</td>
<td>81</td>
</tr>
</tbody>
</table>

P-values:
- P=0.05
- P=0.01
- P=0.006
- P=0.004
Figure 4.2.5  Hsp 70 mean (and 95% CI of mean) with respect to HLA alleles/haplotypes

<table>
<thead>
<tr>
<th>Allele or Haplotype</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1B8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B8DR3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1DR3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Results

Legend to Figures 4.2.4 and 4.2.5

Bar charts of mean Hsp levels (-2SEM to +2SEM) with respect to the presence or absence of individual HLA alleles/haplotypes. P values denote the level of significance for the difference in mean values of Hsp 90 levels in patients with SLE with respect to the presence or absence of HLA alleles/haplotypes., using a Student’s t-test for equality of means. No significant differences in mean Hsp 70 levels were found in patients with SLE with respect to the presence or absence of individual HLA alleles/haplotypes.

Key to Abbreviations:

- n: number of subjects
- A1: HLA A1
- B8: HLA B8
- DR3: HLA DR3
- A1B8: HLA A1, B8
- B8DR3: HLA B8, DR3
- A1DR3: HLA A1, DR3
## Results

**Table 4.2.3** Association of HLA Alleles/Haplotypes with Hsp 90 Elevation in SLE

**Significance Test:** Chi-Square (Pearson)

<table>
<thead>
<tr>
<th>Allele/Haplotype</th>
<th>Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>0.18</td>
<td>0.67</td>
</tr>
<tr>
<td>B8</td>
<td>1.95</td>
<td>0.16</td>
</tr>
<tr>
<td>DR3</td>
<td>4.27</td>
<td>0.04 (significant: p&lt;0.05)</td>
</tr>
<tr>
<td>A1,B8</td>
<td>1.96</td>
<td>0.38</td>
</tr>
<tr>
<td>A1DR3</td>
<td>2.34</td>
<td>0.13</td>
</tr>
<tr>
<td>B8,DR3</td>
<td>1.31</td>
<td>0.25</td>
</tr>
</tbody>
</table>


**Results**

Table 4.2.4  Association of HLA DR3 Status with Hsp 90 Elevation in SLE

<table>
<thead>
<tr>
<th></th>
<th>Hsp 90 Not Elevated</th>
<th>Hsp 90 Elevated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DR3 Present</strong></td>
<td>n = 20</td>
<td>n = 4</td>
</tr>
<tr>
<td></td>
<td>Expected Value 15.9</td>
<td>Expected Value 8.1</td>
</tr>
<tr>
<td></td>
<td>Residual 4.1</td>
<td>Residual -4.1</td>
</tr>
<tr>
<td><strong>DR3 Absent</strong></td>
<td>n = 39</td>
<td>n = 26</td>
</tr>
<tr>
<td></td>
<td>Expected Value 43.1</td>
<td>Expected Value 21.9</td>
</tr>
<tr>
<td></td>
<td>Residual -4.1</td>
<td>Residual 4.1</td>
</tr>
</tbody>
</table>

**Significance Tests**

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chi-Square</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pearson</td>
<td>4.27</td>
<td>0.04</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>- 3.29</td>
<td>0.03</td>
</tr>
<tr>
<td>Linear by Linear Association</td>
<td>- 4.64</td>
<td>0.04</td>
</tr>
<tr>
<td>Fisher's Exact Test:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>One-Tail</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Two-Tail</td>
<td></td>
<td>0.05</td>
</tr>
</tbody>
</table>
4.3 Cytokine Levels in SLE

The mean, 95% confidence limits (-2SEM to +2SEM), median and range of the levels of IL-10 and IL-6 in normals and patients with SLE or RA, are shown in Table 4.3.1. In agreement with previous studies, IL-10 was found to be significantly elevated in patients with SLE (Park et al., 1998) and IL-6 levels were found to be elevated in both patients with SLE (Linker-Israeli et al., 1991) and RA (al-Janadi et al., 1993).

The upper limits of the reference ranges for each cytokine in the control normal group are shown in Table 4.3.2. The 97.5th centile was used as the upper limit of “normal” for both IL-10 and IL-6 (see statistical analyses in Chapter 2, Section 2.6). Table 4.3.3 shows the number and percentage of patients with SLE or RA, with levels of IL-10 or IL-6 above the normal 97.5th centile. Elevated levels of IL-10 were observed in 18 patients with SLE (17%) and two patients with RA (10%). Elevated levels of IL-6 were found in 67 patients with SLE (58%) and 12 patients with RA (60%). Scattergraphs showing the levels of IL-10 and IL-6 in patients with SLE or RA and normal individuals, are shown in Figure 4.3.1 (IL-10) and Figure 4.3.2 (IL-6).

The normal individual with a raised level of IL-10 (IL-10 2.25), was a 38 year old clinically well male, and is the same subject previously determined to have an elevated level of Hsp 90 (Hsp 90 2.6). There were no normal individuals with significantly elevated levels of IL-6.

In view of previous studies (Dhillon et al., 1994) and the results presented here, which support a genetic origin to the overexpression of Hsp 90 in SLE and studies suggesting a role for IL-10 (Ripley et al., 1999) and/or IL-6 (Stephanou et al., 1997; 1998c) in Hsp 90 regulation in SLE, levels of both IL-6 and IL-10 in serum of patients with SLE were compared with the respective patient HLA type. Mean and 95% CI mean (-2SEM to +2SEM) for each cytokine relative to HLA type are shown in Figure 4.3.3 (IL-6) and Figure 4.3.4 (IL-10). No significant differences were found in the mean levels of either cytokine between HLA types in patients with SLE, as determined by a students t-test (see Chapter 2) for equality of means.
### Results

**Table 4.3.1** Cytokine Levels in PBMCs of Normals and Patients with SLE or Rheumatoid Arthritis

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SLE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>116</td>
<td>108</td>
</tr>
<tr>
<td>Mean</td>
<td>1.66</td>
<td>1.94</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.53-1.79</td>
<td>1.83-2.05</td>
</tr>
<tr>
<td>Median</td>
<td>1.55</td>
<td>1.78</td>
</tr>
<tr>
<td>Range</td>
<td>3.90</td>
<td>3.14</td>
</tr>
<tr>
<td>IQR</td>
<td>0.89</td>
<td>0.79</td>
</tr>
<tr>
<td><strong>Normals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Mean</td>
<td>1.01</td>
<td>1.36</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.91-1.12</td>
<td>1.13-1.58</td>
</tr>
<tr>
<td>Median</td>
<td>0.86</td>
<td>1.49</td>
</tr>
<tr>
<td>Range</td>
<td>1.15</td>
<td>2.16</td>
</tr>
<tr>
<td>IQR</td>
<td>0.48</td>
<td>0.52</td>
</tr>
</tbody>
</table>

**P value**< sup>1</sup>  | < 0.01  | < 0.05 |
**SE difference** | 0.08 | 0.15 |
Results

Table 4.3.1 (continued)

Rheumatoid Arthritis

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean</td>
<td>1.88</td>
<td>1.48</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.65-2.12</td>
<td>1.23-1.73</td>
</tr>
<tr>
<td>Median</td>
<td>1.74</td>
<td>1.52</td>
</tr>
<tr>
<td>Range</td>
<td>1.87</td>
<td>2.49</td>
</tr>
<tr>
<td>IQR</td>
<td>1.04</td>
<td>0.98</td>
</tr>
</tbody>
</table>

P value^ <0.02 >0.3
SE difference 0.04 0.17

Key:

P value: Level of significance for the difference in the mean levels of cytokines determined for the control normal group and patients with SLE (P value^) and for the control normal group and patients with rheumatoid arthritis and normals (P value^), using Student’s t-test on raw data.

Nota:

All statistical analyses of levels of IL-6 or IL-10 in all subject groups, presented in this chapter or proceeding chapters, have been performed on log_{10} transformed data (raw data), as discussed in Chapter2. Levels of IL-6 or IL-10 in the raw data were expressed in units of g^5/ml.
**Results**

Table 4.3.2  **Upper Limits of Normal Cytokine Levels and Number of Patients with SLE or Rheumatoid Arthritis with Elevated Levels of Cytokines**

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean + 2SD</td>
<td>1.51</td>
<td>2.41</td>
</tr>
<tr>
<td>97.5(^{th}) centile</td>
<td>1.39</td>
<td>2.08</td>
</tr>
<tr>
<td><strong>SLE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients with elevation above the normal 97.5(^{th}) centile</td>
<td>67</td>
<td>18</td>
</tr>
<tr>
<td>% of patients with elevation</td>
<td>58.00</td>
<td>17.00</td>
</tr>
<tr>
<td><strong>Rheumatoid Arthritis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients with elevation above the normal 97.5(^{th}) centile</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>% of patients with elevation</td>
<td>60.00</td>
<td>10.00</td>
</tr>
</tbody>
</table>
Figure 4.3.1  IL-10 Levels in SLE and Control Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>SLE</th>
<th>Normals</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>108</td>
<td>22</td>
<td>20</td>
</tr>
</tbody>
</table>

Log IL-10 (g/L/ml)
Figure 4.3.2  IL-6 Levels in SLE and Control Groups

Log IL-6 (g⁻⁵/ml)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>116</td>
</tr>
<tr>
<td>Normal</td>
<td>22</td>
</tr>
<tr>
<td>RA</td>
<td>20</td>
</tr>
</tbody>
</table>
Results

Legend to Figures 4.3.1 and 4.3.2

Scattergraphs showing levels of cytokines in patients with SLE or RA and normal individuals
Figure 4.3.3  IL-6 mean (and 95% CI of mean) with respect to HLA alleles/haplotypes in SLE

<table>
<thead>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>DR3</td>
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</tr>
<tr>
<td>A1B8</td>
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<tr>
<td>B8DR3</td>
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</table>
Figure 4.3.4  IL-10 mean (and 95% CI of mean) with respect to HLA alleles/haplotypes

<table>
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<th>Absent</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>
Results

Legend to Figures 4.3.3 and 4.3.4

Bar charts showing the mean levels of cytokines (-2SEM to +2SEM) in patients with SLE with respect to the presence or absence of individual HLA alleles/haplotypes. No significant differences in mean levels of IL-6 or IL-10 were found in patients with SLE with respect to the presence or absence of individual HLA alleles/haplotypes.

Key to Abbreviations:

- n: number of subjects
- A1: HLA A1
- B8: HLA B8
- DR3: HLA DR3
- A1B8: HLA A1, B8
- B8DR3: HLA B8, DR3
- A1DR3: HLA A1, DR3
Results

4.4 Correlation of Cytokine Levels with Heat Shock Protein Levels in SLE

In this chapter, levels of cytokines and Hsps have been determined respectively in individual serum and PBMC samples, which were isolated at the same time from normal subjects and patients with SLE or RA. An investigation of the relative effects of IL-6 and IL-10 on the regulation of Hsp 90 and Hsp 70 gene expression in SLE, is outlined in this section.

Levels of cytokines were compared with levels of Hsps in patients with SLE. Figure 4.4.1 is a scatter graph of IL-6 levels compared with Hsp 90 levels in patients with SLE and shows a significant correlation between the two variables ($r=0.39$, $P<0.05$). No significant correlation was found between levels of IL-6 and Hsp 70 in patients with SLE (Figure 4.4.2). Levels of IL-10 did not correlate with levels of Hsp 90 (Figure 4.4.3) or with levels of Hsp 70 (Figure 4.4.4), in patients with this disease.

Mean levels of Hsp 90 were compared in patients with elevated to those with non-elevated levels of IL-6. Figure 4.4.5 shows that patients with elevated levels of IL-6 had significantly higher mean levels of Hsp 90 in comparison with patients with non-elevated IL-6 levels ($P<0.05$) and the total SLE patient cohort ($P<0.1$).

The reverse comparison of IL-6 levels in patients with elevated and non-elevated Hsp 90 is shown in Figure 4.4.6. Significantly higher mean IL-6 levels are found in patients with elevated Hsp 90 compared to both patients with non-elevated Hsp 90 and the total studied SLE population. Interestingly, the differences in mean IL-6 levels between those patients with elevated and non-elevated Hsp 90 are significant much more than that found for the differences in mean Hsp 90.
Figure 4.4.1 shows the correlation between levels of IL-6 and Hsp 90 in individual samples from patients with SLE. A trendline and the associated correlation coefficient ($r=0.39$) and Pearson’s $p$ value ($p<0.01$) are also shown.
Figure 4.4.2 shows the correlation between levels of IL-6 and Hsp 70 in individual samples from patients with SLE. A trendline with the associated correlation coefficient ($r=-0.11$) is also shown.
Figure 4.4.3 shows the correlation between levels of IL-10 and Hsp 90 in individual samples from patients with SLE. A trendline with the associated correlation coefficient ($r = -0.08$) is also shown.
Figure 4.4.4  Correlation of Between Levels of IL-10 and Hsp 70 in Individual Samples from Patients with SLE

Hsp 70

Log_{10} IL-10 (g^5/ml)

Figure 4.4.4 is a scattergraph showing the correlation between levels of IL-10 and Hsp 70 in individual samples from patients with SLE. A trendline and associated correlation coefficient (r=-0.16) is also shown.
Figure 4.4.5  Hsp 90 Mean (and 95% CI of Mean) with Respect to IL-6 Levels in Normals and SLE

P<0.10

P<0.05
Figure 4.4.6  IL-6 Mean (and 95% CI of Mean) with Respect to Hsp 90 Levels in Normals and SLE

Log₁₀ IL-6 (g⁻³/ml)

- P < 0.05
- P < 0.02

<table>
<thead>
<tr>
<th>n</th>
<th>30 Normals</th>
<th>95 All SLE</th>
<th>67 Hsp 90 normal</th>
<th>28 Hsp 90 elevated</th>
</tr>
</thead>
</table>
Results

Legend to Figure 4.4.5

Bar chart showing the mean levels of Hsp 90 (-2SEM to +2SEM) in the normal control group and in the total SLE patient population and patients with normal or elevated levels of IL-6. P values represent the level of significance for the difference in mean Hsp 90 levels between groups, using a students t-test for the equality of means.

Legend to Figure 4.4.6

Bar chart showing the mean levels of IL-6 (-2SEM to +2SEM) in the normal control group and in the total SLE patient population and patients with normal or elevated levels of Hsp 90. P values represent the level of significance for the difference in mean IL-6 levels between groups, using a students t-test for the equality of means.
Results

levels between patients with elevated and non-elevated IL-6 levels ($0.02 < P < 0.05$ compared with $0.05 < P < 0.1$, respectively). A scatter graph comparing IL-6 and Hsp 90 levels in patients who overexpress Hsp 90 is shown in Figure 4.4.7. Levels of IL-6 correlated with Hsp 90 levels ($r = 0.49$, $P < 0.01$) and the degree of correlation was found to be much higher than that found (Figure 4.4.1) in a comparison between IL-6 and Hsp 90 levels for the whole population of SLE patients ($r = 0.39$, $P < 0.01$).
**Figure 4.4.7** Correlation of IL-6 and Hsp 90 in Patients with SLE who Overexpress Hsp 90

Figure 4.4.7 shows the correlation between levels of IL-6 and Hsp 90 in individual samples from patients with SLE who overexpress Hsp 90. A trendline and the associated correlation coefficient ($r=0.49$) and Pearson's $p$ value ($p<0.01$) are also shown.
4.5 Discussion

A role for Hsps in the pathogenesis of autoimmune diseases has been extensively studied (see Chapter 1, Section 1.2) and may involve the induction of Hsp-specific immune responses, with pathological consequences. Studies of Hsp expression in patients with SLE, have previously highlighted the specific overexpression of Hsp 90 in this disease, in the absence of elevation of other Hsp family members (Dhillon et al., 1993a). In agreement with these observations, results in this chapter have demonstrated normal levels of Hsp 70 in SLE and elevated levels of Hsp 90 in approximately 30% of patients with SLE. Furthermore, levels of Hsp 70 and Hsp 90 were not elevated in patients with rheumatoid arthritis. These results therefore support a previously held theory which suggests Hsp 90 overexpression in SLE to represent a distinct regulatory mechanism specific to the induction of Hsp 90 and not a general cellular response to stress (Latchman and Isenberg, 1994). However, until now, an understanding of the molecular events which lead to Hsp 90 overexpression has been unresolved.

SLE is characterised by B cell hyperactivity, reflected in a broad spectrum of autoantibodies and decreased in vivo and in vitro cellular immune responses. Such immune responses may in part reflect abnormalities in the expression of cytokines, which regulate the function of cells within the normal immune system. Elevated levels of the cytokines IL-6 (Linker-Israeli et al., 1991) and IL-10 (Park et al., 1998) are found in patients with active SLE and a number of studies have suggested a role for these cytokines in the overexpression of Hsp 90 in SLE. Thus, IL-6 (Stephanou et al., 1997) and IL-10 (Ripley et al., 1999) (see Chapter 3) have been demonstrated to activate Hsp 90 expression in vitro and Hsp 90 is specifically elevated in IL-6 transgenic mice (Stephanou et al., 1998a).

The relative effects of these two factors in the overexpression of Hsp 90 in SLE has been investigated in this chapter. Levels of IL-6 were found to correlate significantly with levels of Hsp 90 but not Hsp 70 in patients with SLE. In contrast, levels of IL-10 did not correlate with either Hsp 90 or Hsp 70 levels in this disease. Thus, these observations support the involvement of a distinct regulatory mechanism specific for the overexpression of Hsp 90 in SLE and one which is mediated by
Results

elevated levels of IL-6 and not elevated levels of IL-10, in patients with this disease. Of particular interest to the studies in this chapter, is the observation of an increased correlation between levels of IL-6 and Hsp 90 in patients who overexpress Hsp 90.

A genetic association with Hsp 90 overexpression in SLE has been suggested by the results presented in this chapter, which show that those patients who lack the HLA B8 or DR3 alleles, are those most likely to overexpress Hsp 90. As discussed in Chapter 1, Section 3, the gene encoding Hsp 90β is located on Chromosome 6 (Durkin et al., 1993; Takahashi et al., 1994), the site of the MHC. It is conceivable that polymorphisms exist within the promoter region of the Hsp 90β gene, which in a subset of patients with SLE, contribute to the observed elevated expression of Hsp 90. Genetic upregulation of Hsp 90 protein in these patients might occur via an increase in the basal level of Hsp 90 gene expression, or indeed be mediated by an enhanced transcriptional response to elevated levels of IL-6 in SLE. However, to date, no studies have established the presence of polymorphisms within regulatory regions of the Hsp 90β gene.

In criticism of the association of elevated Hsp 90 expression in SLE with patients lacking HLA B8 and/or DR3 alleles, is the absence of statistical analyses which compare Hsp 90 expression in different ethnic groups of both the total SLE population and of the control normal population. Such analyses are of importance since, as discussed in Chapter 1, Section 1.3, different HLA alleles occur with varying frequencies in different populations, according to both ethnic composition and geographic location. However, since the number of patients in some ethnic groups was low, statistical analyses of this type are unlikely to attain significance. The study of Dhillon et al., (1994), also found an association between elevated Hsp 90 levels in SLE and patients lacking HLA B8 and/or DR3 alleles. This study used a greater number of SLE patients (incorporating the study of Norton et al., 1989) and a population of similar ethnic composition (see Chapter 2) to that used in this dissertation. No differences in Hsp 90 levels were observed in White Caucasoid SLE patients compared with Non-White patients, suggesting these findings and possibly those of this dissertation, to not be the result of ethnic bias.

The overexpression of Hsp 90 in SLE may therefore be mediated both by elevated levels of IL-6 in this disease and genetic factors, which for significantly
Results

elevated levels of IL-6, sustain a linear regulatory relationship between IL-6 and Hsp 90. Taken together, these results suggest an involvement of IL-6 and not IL-10 in the regulation of Hsp 90 in SLE, which is enhanced in a genetic subset of patients with SLE.
Chapter 5
Elevated Expression of Hsp 90
and Antibodies to Hsp 90 in SLE

5.0 Introduction

A role for heat shock proteins (Hsps) in the pathogenesis of autoimmune disease has been extensively reviewed. These studies have highlighted possible mechanisms by which Hsps can initiate an autoimmune response (discussed in Chapter 1, Section 1.2). For example, the mycobacterial Hsp 65 may play a role in adjuvant arthritis in rats and increased levels of antibodies to Hsp 65 have been reported in patients with rheumatoid arthritis (Tsoulfa et al., 1989).

Overexpression of the 90 kDa heat shock protein (Hsp 90) is evident in a subset of patients with SLE (Dhillon et al., 1994) and antibodies to Hsp 90 have been observed in approximately one-third of patients with SLE, of both adult (Conroy et al., 1994) and childhood onset (Conroy et al., 1996). Any role for Hsp 90 in the development of Hsp 90 autoantibodies is likely to require its localisation on the surface of cells of the immune system, where it is accessible to the immune system (discussed in Chapter 1, Section 1.4).

Surface expression of Hsp 90 in peripheral blood mononuclear cells (PBMCs) from patients with SLE, has been investigated by flow-cytometry (Erkeller-Yuksel et al., 1992). In this study, approximately 20% of patients with SLE were found to surface express Hsp 90, but this was rarely observed in patients with rheumatoid arthritis or Sjogren’s disease. Furthermore, surface expression of Hsp 90 in SLE, correlated with the specific subset of patients in which overexpression of Hsp 90 is a feature. A role for anti-Hsp 90 antibodies in the pathogenesis of SLE, is supported by studies in a murine model of lupus. In autoimmune MRL-Ipr/lpr mice, the
Results

development of antibodies to Hsp 90 precedes the onset of lupus-like disease and occurs after splenocyte elevation of Hsp 90 (Faulds et al., 1994). Moreover, antibodies to Hsp 90 have been identified in 60% of MRL-\textit{lpr/lpr} mice and were rarely found in control BALB/C mice (Faulds et al., 1995).

Studies in murine models of SLE therefore suggest a role for Hsp 90 overexpression in the development of antibodies to Hsp 90 in this disease (reviewed in Stephanou et al., 1998c). Since the presence of these antibodies precedes the onset of disease in MRL-\textit{lpr/lpr} mice, an understanding of the mechanisms which lead to their production in human SLE, are of great interest. Interestingly, elevation of IL-6 in transgenic mice, leads to increased levels of Hsp 90 and the production of anti-Hsp 90 antibodies (Stephanou et al., 1998a).

In the previous chapter, a number of patients with SLE were identified who overexpressed Hsp 90 and these patients correlated with a subset of patients which has been reported as being most likely to overexpress Hsp 90 (Dhillon et al., 1994). Using serum samples taken from normal individuals and patients with SLE, isolated at the time of Hsp quantitation, an investigation of the correlation between Hsp overexpression and the presence of raised levels of antibodies to Hsps in SLE, is now possible and is detailed in this chapter.
Results

5.1 Detection of Antibodies to Hsps

Using serum samples from normal individuals and patients with SLE or rheumatoid arthritis (RA), levels of IgG and IgM antibodies to Hsp 90 and Hsp 70, were measured by ELISA, using the methods outlined in Chapter 2. Serum samples obtained from individual patients with SLE or RA, were considered to have a raised level of an antibody (IgG or IgM to Hsp 90 or Hsp 70) if the level was above the 97.5\textsuperscript{th} centile of the respective antibody levels in the normal control group.

5.2 Pattern of Expression of Antibodies to Hsps in SLE

As shown in Table 5.2.1 and Figure 5.2.1, raised levels of IgG antibodies to Hsp 90 were detected in 46\% of patients with SLE. Only 15\% of patients with RA were found to have raised levels of IgG antibodies to Hsp 90. Raised IgM antibody levels to Hsp 90 (Table 5.2.1 and Figure 5.2.2) were detected in 28\% of patients with SLE and 10\% of patients with RA. Thus, raised levels of antibodies (IgG or IgM) to Hsp 90 were frequently detected in patients with SLE and in contrast, were rarely detected in patients with RA.

Table 5.2.1 also shows the number and percentage of patients with SLE or RA with raised levels of antibodies to Hsp 70. In patients with SLE, raised levels of IgG antibodies to Hsp 70 (Figure 5.2.3) were detected in 14\% of subjects. Only 5\% of patients with RA were found to have raised IgG levels to Hsp 70. Levels of IgM antibodies to Hsp 70 (Figure 5.2.4) were detected in 18\% of patients with SLE and were not detected in patients with RA.

Taken together, the expression pattern of antibodies to Hsp 90 and Hsp 70 in patients with SLE or RA presented here, is in agreement with previous studies of patients with SLE (Conroy et al., 1994; 1996) and which used an identical ELISA system for antibody detection to that used in the studies in this chapter.
Results

Table 5.2.1  Upper Limits of Normal Levels of Antibodies to HSPs and Number of Patients with SLE or RA with Raised Antibody Levels

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<thead>
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<th>Antibody to Hsp</th>
<th>Hsp 90 IgG</th>
<th>Hsp 90 IgM</th>
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<tr>
<td><strong>Normals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Mean +2SD</td>
<td>0.54</td>
<td>0.47</td>
</tr>
<tr>
<td>97.5&lt;sup&gt;th&lt;/sup&gt; Centile</td>
<td>0.61</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>SLE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>116</td>
<td>102</td>
</tr>
<tr>
<td>Mean antibody level</td>
<td>0.71</td>
<td>0.42</td>
</tr>
<tr>
<td>Number of patients with raised antibody level (above the normal 97.5&lt;sup&gt;th&lt;/sup&gt; centile)</td>
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<td>29</td>
</tr>
<tr>
<td>% of patients with raised antibody level</td>
<td>46</td>
<td>28</td>
</tr>
<tr>
<td><strong>Rheumatoid Arthritis</strong></td>
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<td></td>
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<tr>
<td>n</td>
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<td>20</td>
</tr>
<tr>
<td>Mean antibody level</td>
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<td>0.33</td>
</tr>
<tr>
<td>Number of patients with raised antibody level (above the normal 97.5&lt;sup&gt;th&lt;/sup&gt; centile)</td>
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<td>2</td>
</tr>
<tr>
<td>% of patients with raised antibody level</td>
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<td>10</td>
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**Results**

**Table 5.2.1 (continued)**

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<th>Hsp 70 IgG</th>
<th>Hsp 70 IgM</th>
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<td><strong>Normals</strong></td>
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<td>n</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Mean +2SD</td>
<td>1.1</td>
<td>0.61</td>
</tr>
<tr>
<td>97.5(^{th}) Centile</td>
<td>1.2</td>
<td>0.53</td>
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<tr>
<td><strong>SLE</strong></td>
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<td></td>
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<tr>
<td>n</td>
<td>98</td>
<td>102</td>
</tr>
<tr>
<td>Mean antibody level</td>
<td>1.13</td>
<td>0.49</td>
</tr>
<tr>
<td>Number of patients with raised antibody level (above the normal 97.5(^{th}) centile)</td>
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<td>12</td>
</tr>
<tr>
<td>% of patients with raised antibody level</td>
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<td>18</td>
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<tr>
<td><strong>Rheumatoid Arthritis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean antibody level</td>
<td>0.62</td>
<td>0.33</td>
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<tr>
<td>Number of patients with raised antibody level (above the normal 97.5(^{th}) centile)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>% of patients with raised antibody level</td>
<td>5</td>
<td>0</td>
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</table>
Figure 5.2.1  Levels of IgG Antibodies to Hsp 90 in SLE and Control Groups
Figure 5.2.2  Levels of IgM Antibodies to Hsp 90 in SLE and Control Groups
Figure 5.2.3  Levels of IgG Antibodies to Hsp 70 in SLE and Control Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>98 SLE</th>
<th>30 Normals</th>
<th>20 RA</th>
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</thead>
<tbody>
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<td>n</td>
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</table>
Figure 5.2.4 Levels of IgM Antibodies to Hsp 70 in SLE and Control Groups
Results

Legend to Figures 5.2.1-5.2.4

Scatter graphs comparing the levels of anti-Hsp antibodies in patients with SLE, normal individuals and patients with rheumatoid arthritis. A solid horizontal black line indicates an antibody level corresponding to the 97.5\textsuperscript{th} centile of the antibody levels in the normal control group.

Key

\begin{tabular}{ll}
\textbf{n} & number of subjects \\
\textbf{SLE} & systemic lupus erythematosus patient group \\
\textbf{RA} & rheumatoid arthritis patient group \\
\end{tabular}
Results

5.3 Immunogenetic Analysis of Antibodies to Hsps

Previous studies have suggested a genetic association for Hsp 90 overexpression (Dhillon et al., 1994) and these observations are in agreement with the results presented in Chapter 4. These studies suggest that elevated levels of Hsp 90 in SLE are most likely to occur in a genetic subset of patients with SLE who have an HLA DR3 negative haplotype. An immunogenetic analysis of antibodies to Hsps in SLE has also been reported (Conroy et al., 1994) and is discussed in Chapter 1, Section 1.4. In this study, a correlation between the presence of raised levels of anti-Hsp 90 antibodies and patients who have an HLA DR3 negative haplotype, was observed. Missing from these studies however, has been a comparative immunogenetic analysis of both elevated Hsp 90 protein levels and raised anti-Hsp 90 antibody levels, in the same patients.

Tables 5.3.1-4 show the number of patients with SLE who were determined to have raised (IgG or IgM) anti-Hsp 90 or anti-Hsp 70 antibodies, with respect to the presence or absence of individual/combined HLA haplotypes. As shown in Table 5.3.1, raised IgG anti-Hsp 90 antibodies were found to correlate with patients who lack the HLA DR3 haplotype (p = 0.043), as determined by a chi-square analysis (described in Chapter 2) and is in agreement with the study of (Conroy et al., 1994). Interestingly, the absence of this haplotype in patients with SLE, was observed (see Chapter 4) to correlate significantly (p = 0.042) with elevated (above the normal 97.5th centile) Hsp 90 levels, as determined by chi-square analysis. No significant correlations were found in comparisons between either IgM anti-Hsp 90 antibodies or anti-Hsp 70 antibodies (IgG or IgM) and the presence or absence of individual/combined HLA haplotypes and this is in agreement with the study of (Conroy et al., 1994).

The observation of shared immunogenetic associations, both for Hsp 90 overexpression and raised antibodies to Hsp 90, in individual patients with SLE, suggests a common link between these events. Thus, it was thought to be of interest to compare levels of Hsp 90 and levels of antibodies to Hsp 90, in individual patients with SLE.


**Results**

Table 5.3.1  Immunogenetic Analysis of IgG Anti-Hsp 90 Antibodies in SLE

<table>
<thead>
<tr>
<th>HLA Type</th>
<th>Hsp 90 Negative</th>
<th>Hsp 90 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1+</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>A1-</td>
<td>37</td>
<td>30</td>
</tr>
<tr>
<td>B8+</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>B8-</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>DR3+</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>DR3-</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>A1, B8+</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>A1, B8-</td>
<td>48</td>
<td>47</td>
</tr>
<tr>
<td>A1, DR3+</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>A1, DR3-</td>
<td>47</td>
<td>44</td>
</tr>
<tr>
<td>B8, DR3+</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>B8, DR3-</td>
<td>49</td>
<td>49</td>
</tr>
</tbody>
</table>

**Key:**

NS  Not Significant
### Results

**Table 5.3.2 Immunogenetic Analysis of IgM Anti-Hsp 90 Antibodies in SLE**

<table>
<thead>
<tr>
<th>HLA Type</th>
<th>Hsp 90 Negative</th>
<th>Hsp 90 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1+</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>A1-</td>
<td>39</td>
<td>29</td>
</tr>
<tr>
<td>B8+</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>B8-</td>
<td>45</td>
<td>41</td>
</tr>
<tr>
<td>DR3+</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>DR3-</td>
<td>36</td>
<td>27</td>
</tr>
<tr>
<td>A1, B8+</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>A1, B8-</td>
<td>49</td>
<td>43</td>
</tr>
<tr>
<td>A1, DR3+</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>A1, DR3-</td>
<td>44</td>
<td>41</td>
</tr>
<tr>
<td>B8, DR3+</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>B8, DR3-</td>
<td>51</td>
<td>43</td>
</tr>
</tbody>
</table>

**Key**

- NS Not Significant
## Results

Table 5.3.3  Immunogenetic Analysis of IgG Anti-Hsp 70 Antibodies in SLE

<table>
<thead>
<tr>
<th>HLA Type</th>
<th>Hsp 70 Negative</th>
<th>Hsp 70 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1+</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>A1-</td>
<td>35</td>
<td>33</td>
</tr>
<tr>
<td>B8+</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>B8-</td>
<td>47</td>
<td>36</td>
</tr>
<tr>
<td>DR3+</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>DR3-</td>
<td>39</td>
<td>27</td>
</tr>
<tr>
<td>A1, B8+</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>A1, B8-</td>
<td>47</td>
<td>42</td>
</tr>
<tr>
<td>A1, DR3+</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>A1, DR3-</td>
<td>46</td>
<td>45</td>
</tr>
<tr>
<td>B8, DR3+</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>B8, DR3-</td>
<td>48</td>
<td>42</td>
</tr>
</tbody>
</table>

**Key:**

NS  Not Significant
### Table 5.3.4 Immunogenetic Analysis of IgM Anti-Hsp 70 Antibodies in SLE

<table>
<thead>
<tr>
<th>HLA Type</th>
<th>Hsp 70 Negative</th>
<th>Hsp 70 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1+</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>A1-</td>
<td>38</td>
<td>34</td>
</tr>
<tr>
<td>B8+</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>B8-</td>
<td>44</td>
<td>41</td>
</tr>
<tr>
<td>DR3+</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>DR3-</td>
<td>37</td>
<td>28</td>
</tr>
<tr>
<td>A1, B8+</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>A1, B8-</td>
<td>49</td>
<td>44</td>
</tr>
<tr>
<td>A1, DR3+</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>A1, DR3-</td>
<td>45</td>
<td>46</td>
</tr>
<tr>
<td>B8, DR3+</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>B8, DR3-</td>
<td>48</td>
<td>46</td>
</tr>
</tbody>
</table>

**Key:**

NS  Not Significant
5.4 Correlation of Hsp Levels with Antibodies to Hsps in SLE

Levels of serum antibodies to Hsp 90 and Hsp 70 were compared with their respective Hsp protein levels in PBMCs, obtained from individual patients with SLE, using the methods described in Chapter 2.

As shown in Table 5.4.1 and Table 5.4.2, raised IgG antibody levels to Hsp 90 in individual patients with SLE, correlated with elevated Hsp 90 levels in the same patients (p=0.018), as determined by a chi-square analysis (see Table 5.4.2). In contrast, Table 5.4.1 shows that levels of Hsp 90 were not found to correlate with levels of IgM antibodies to Hsp 90. Scattergraphs comparing levels of Hsp 90 with antibodies to Hsp 90 are shown in Figure 5.4.1 (IgG antibodies to Hsp 90) and Figure 5.4.2 (IgM antibodies to Hsp 90). Table 5.4.1 shows that correlations between levels of Hsp 70 and raised antibody levels to Hsp 70 (IgG or IgM) in patients with SLE, were not found to be significant, as determined by chi-square analyses. Scattergraphs comparing levels of Hsp 70 with antibodies to Hsp 70 are shown in Figure 5.4.3 (IgG antibodies to Hsp 70) and Figure 5.4.4 (IgM antibodies to Hsp 70).

In previous studies, elevated levels of Hsp 90 (Dhillon et al., 1993a) and raised levels of antibodies to Hsp 90 (Conroy et al., 1994) have been observed in SLE. However, any correlation between elevated levels of cytoplasmic Hsp 90 and the presence of raised levels of anti-Hsp 90 antibody in SLE, has remained unproven. A significant correlation between elevated Hsp 90 levels and raised levels of IgG antibodies to Hsp 90 in patients with SLE has been detailed above and this is the first time that such a correlation has been shown.
**Results**

Table 5.4.1  Number of Patients with SLE with Elevated Hsp90 and Raised Hsp Antibody Levels

<table>
<thead>
<tr>
<th>Protein Levels</th>
<th>IgG Antibodies to Hsp 90</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Levels</td>
<td>Raised Levels</td>
<td></td>
</tr>
<tr>
<td>Elevated Hsp 90</td>
<td>12</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Non-Elevated Hsp 90</td>
<td>39</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

- \( P = 0.018 \)

<table>
<thead>
<tr>
<th>Protein Levels</th>
<th>IgM Antibodies to Hsp 90</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal Levels</td>
<td>Raised Levels</td>
<td></td>
</tr>
<tr>
<td>Elevated Hsp 90</td>
<td>21</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Non-Elevated Hsp 90</td>
<td>41</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

NS
Results

Table 5.4.1 (continued)

<table>
<thead>
<tr>
<th>Protein Levels</th>
<th>IgG Antibodies to Hsp 70</th>
<th>Normal Levels</th>
<th>Raised Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated Hsp 70</td>
<td></td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Non-Elevated Hsp 70</td>
<td></td>
<td>89</td>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein Levels</th>
<th>IgM Antibodies to Hsp 70</th>
<th>Normal Levels</th>
<th>Raised Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated Hsp 70</td>
<td></td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Non-Elevated Hsp 70</td>
<td></td>
<td>81</td>
<td>14</td>
</tr>
</tbody>
</table>
**Results**

Table 5.4.2  Chi-Square Test for the Correlation of Hsp 90 Overexpression with Raised IgG Antibodies to Hsp 90 in SLE

<table>
<thead>
<tr>
<th></th>
<th>Normal Hsp 90</th>
<th>Elevated Hsp 90</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody -ve</td>
<td>39</td>
<td>12</td>
<td>51</td>
</tr>
<tr>
<td>Antibody +ve</td>
<td>20</td>
<td>18</td>
<td>38</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>59</strong></td>
<td><strong>30</strong></td>
<td></td>
</tr>
</tbody>
</table>

Chi-Square Significance

- Pearson: 0.018
- Likelihood Ratio: 0.018
- Linear by Linear Association: 0.019
- Fisher's Exact Test: 0.016 One-tail, 0.024 Two-tail
Figure 5.4.1  Correlation of Hsp 90 with IgG Antibodies to Hsp 90 In SLE
Figure 5.4.2 Correlation of Hsp 90 with IgM Antibodies to Hsp 90
Figure 5.4.3  Comparison of Levels of Hsp 70 with Levels of IgG Antibodies to Hsp 70 in SLE
Figure 5.4.4 Comparison of Levels of Hsp 70 with Levels of IgM Antibodies to Hsp 70 in SLE
Results

Legend to Figures 5.4.1-5.4.4

Scatter graphs comparing the levels of anti-Hsp antibodies and Hsps in patients with SLE.

Key

SLE  systemic lupus erythematosus patient group
RA   rheumatoid arthritis patient group
Results

5.5 Correlation of Levels of Cytokines and Antibodies to Hsps in SLE

As discussed in Chapter 1, Section 1.3, SLE is characterised by B cell hyperactivity and decreased cell mediated immunity. Such immune disturbances may be partly due to abnormalities in the expression of cytokines in this disease. For example, elevated levels of the cytokines IL-6 (Linker-Israeli et al., 1991) and IL-10 (Park et al., 1998) have been reported in SLE. Moreover, as discussed in Chapter 1, Section 1.3, the stimulatory effects of both IL-6 and IL-10 on B cell immunoglobulin synthesis together with the known inhibitory effects of IL-10 on cell-mediated immune responses, may in part explain these immunological anomalies in patients with SLE.

The studies detailed in Chapter 4, have shown a significant correlation between elevated levels of IL-6 but not IL-10, on Hsp 90 overexpression in patients with SLE. However, a general stimulatory effect of either IL-6 and/or IL-10 on B cells, may also be involved in the production of anti-Hsp antibodies, including those with specificity for Hsp 90. Levels of both cytokines have therefore been compared with levels of anti-Hsp antibodies, in samples from individual patients with SLE.

Scatter graphs comparing the levels of IL-6 with levels of anti-Hsp 90 antibodies are shown in Figure 5.5.1 (IgG isotype) and Figure 5.5.2 (IgM isotype). No significant correlation was found between levels of IL-6 and anti-Hsp 90 antibodies (see Table 5.5.1). Scatter graphs showing a comparison between levels of IL-6 and levels of anti-Hsp 70 antibodies in patients with SLE are shown in Figure 5.5.3 (IgG isotype) and Figure 5.5.4 (IgM isotype). No significant correlation was found between levels of IL-6 and anti-Hsp 70 antibodies (see Table 5.5.1). A similar comparison of IL-10 levels and levels of anti-Hsp antibodies is outlined below.

Scatter graphs comparing the levels of IL-10 with levels of anti-Hsp 90 antibodies are shown in Figure 5.5.5 (IgG isotype) and Figure 5.5.6 (IgM isotype). No significant correlation was found between levels of IL-10 and anti-Hsp 90 antibodies (see Table 5.5.2). Scatter graphs showing a comparison between levels of IL-10 and levels of anti-Hsp 70 antibodies in patients with SLE are shown in Figure
Results

5.5.7 (IgG isotype) and Figure 5.5.8 (IgM isotype). Similarly, no significant correlation was found between levels of IL-10 and anti-Hsp 70 antibodies (see Table 5.2).
Results

Table 5.5.1  Correlation Between Levels of IL-6 and Antibodies to Hsps in SLE

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Correlation Coefficient</th>
<th>Significance (Pearson)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp 90 IgG</td>
<td>( r=0.08 )</td>
<td>NS</td>
</tr>
<tr>
<td>Hsp 90 IgM</td>
<td>( r=0.04 )</td>
<td>NS</td>
</tr>
<tr>
<td>Hsp 70 IgG</td>
<td>( r=0.12 )</td>
<td>NS</td>
</tr>
<tr>
<td>Hsp 70 IgM</td>
<td>( r=0.16 )</td>
<td>NS</td>
</tr>
</tbody>
</table>

Legend to Table 5.5.1

Correlations were performed using the methods outlined in Altman (1999). Antibody level data were categorised as binary variables and coded as normal (code=0) or elevated (code=1), as defined earlier in this chapter and compared with levels of IL-6 as a continuous variable. A correlation coefficient and Pearson significance was derived. P values were considered significant if below the 5% level (p<0.05). As shown above, no significant associations were found.

Key:

NS not significant
Figure 5.5.1  Comparison of IL-6 Levels with Levels of IgG Antibodies to Hsp 90 in SLE
Figure 5.5.2  Comparison of IL-6 Levels with Levels of IgM Antibodies to Hsp 90 in SLE
Figure 5.5.3 Comparison of IL-6 Levels with Levels of IgG Antibodies to Hsp 70 In SLE
Figure 5.5.4 Comparison of IL-6 and IgM Antibodies to Hsp 70 in SLE
Results

Legend to Figures 5.5.1- 5.5.4

Scatter graphs comparing the levels of IL-6 and levels of antibodies to Hsps in patients with SLE.
### Results

**Table 5.5.2** Correlation Between Levels of IL-10 and Antibodies to Hsps in SLE

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Correlation Coefficient</th>
<th>Significance (Pearson)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp 90 IgG</td>
<td>r=0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Hsp 90 IgM</td>
<td>r=0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Hsp 70 IgG</td>
<td>r=0.04</td>
<td>NS</td>
</tr>
<tr>
<td>Hsp 70 IgM</td>
<td>r=0.13</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Legend to Table 5.5.2**

Correlations were performed using the methods outlined in Altman (1999). Antibody level data were categorised as binary variables and coded as normal (code=0) or elevated (code=1), as defined earlier in this chapter and compared with levels of IL-6 as a continuous variable. A correlation coefficient and Pearson significance was derived. P values were considered significant if below the 5% level (p<0.05). As shown above, no significant associations were found.

**Key:**

NS not significant
Figure 5.5.5  Comparison of Levels of IL-10 with Levels of IgG Antibodies to Hsp 90 in SLE
Figure 5.5.6 Comparison of Levels of IL-10 with Levels of IgM Antibodies to Hsp 90 in SLE
Figure 5.5.7 Comparison of Levels of IL-10 with Levels of IgG Antibodies to Hsp 70 in SLE
Figure 5.5.8 Comparison of Levels of IL-10 with Levels of IgM Antibodies to Hsp 70 in SLE
Results

Legend to Figures 5.5.5- 5.5.8

Scatter graphs comparing the levels of IL-10 and levels of antibodies to Hsp in patients with SLE.
5.6 Discussion

Antibodies to both Hsp 90 and Hsp 70 have been reported in patients with SLE (Conroy et al., 1994; 1996). Interestingly, an immunogenetic analysis of anti-Hsp 90 antibodies in SLE (Conroy et al., 1994) correlates with a genetic subset of patients most likely to overexpress Hsp 90 protein (Dhillon et al., 1994). A link between Hsp 90 overexpression and the development of anti-Hsp 90 antibodies has been suggested (Stephanou et al., 1998c) and may involve the altered presentation of Hsp 90 to the immune system. In agreement with this, cell surface expression of Hsp 90 has been detected in lymphocytes from patients with SLE (Erkeller-Yuksel et al., 1992). However, until now, a study comparing Hsp 90 protein levels and levels of anti-Hsp 90 antibodies from the same patients, has not been undertaken.

A significant correlation between Hsp 90 overexpression in PBMCs from patients with SLE and the presence of IgG anti-Hsp 90 antibodies in the serum of these patients was found ($p = 0.018$). No such correlation was found between Hsp 70 and anti-Hsp 70 antibodies, in patients with SLE. Since protein levels of Hsp 90 and not Hsp 70 were found to be significantly elevated in these patients (see Chapter 4), these observations are in agreement with the aforementioned hypothesis which linked the differential expression and surface localisation of Hsps in SLE, with the development of anti-Hsp 90 antibodies (Stephanou et al., 1998c). Interestingly, observations of anti-Hsp 90 antibodies in SLE, parallels studies in autoimmune MRL/lpr mice, in which splenocyte elevation of Hsp 90 (Faulds et al., 1994) and autoantibodies to Hsp 90 (Faulds et al., 1995), preceded the onset of disease. Furthermore, studies of human breast cancer have demonstrated elevated Hsp 90 levels (Jameel et al., 1992) and autoantibodies to Hsp 90 [Conroy, 1995 #35 in this disease.

A role for cytokines in the pathogenesis of SLE has been suggested and is supported by the observations that levels of IL-6 (Linker-Israeli et al., 1991) and IL-10 (Park et al., 1998) are elevated in this disease. Such cytokine abnormalities may contribute to increased antibody and decreased cell-mediated immune responses observed in this disease (Linker-Israeli, 1992). Although a role for IL-6 and not IL-10 in the overexpression of Hsp 90 in SLE, has been detailed in Chapter 4, an analysis
Results

of the involvement of either cytokine in the development of anti-Hsp antibody production as part of a general B cell stimulatory effect, has until now not been investigated. The results in this chapter show that levels of antibodies to Hsp 90 and Hsp 70 did not correlate with either IL-6 or IL-10 levels. Thus it is likely that any role for IL-6 in the development of anti-Hsp 90 antibodies in this disease, is mediated most strongly by the effect of IL-6 on elevated Hsp 90 levels in this disease.
Chapter 6
Disease Associations

6.1 Introduction

Our understanding of the pathogenesis of systemic lupus erythematosus (SLE) has been aided by the recognition of a broad spectrum of immunological abnormalities and clinical manifestations which characterise this disease (see Chapter 1, Section 1.3). Of particular interest to the studies in this dissertation has been the role of cytokines in the expression of heat shock proteins (Hsps) in SLE and the development of antibodies to Hsps, with possible pathological consequences.

As discussed in detail in Chapter 1, Section 1.4 and reviewed in Stephanou et al., (1998c), previous studies have reported the overexpression of Hsp 90 and raised levels of antibodies to Hsp 90 in patients with SLE and these observations are in agreement with the results presented in this dissertation. These studies have been extended (see Chapter 4) to show that overexpression of Hsp 90 in SLE, correlated with elevated levels of the cytokine IL-6 but not IL-10, in patients with this disease. Furthermore, in individual patients with SLE, raised levels of IgG antibodies to Hsp 90 were most strongly associated with elevated levels of Hsp 90 in the same patients (see Chapter 5). Although various studies suggest a role for Hsps (discussed in Chapter 1, Section 1.2) and cytokines (discussed in Chapter 1, Section 1.3) in SLE, the relative contribution of these factors to clinical disease has remained unclear.

The British Isles Lupus Assessment Group (BILAG) index is a computerised index for measuring clinical disease activity in patients with SLE (Symmons et al., 1988). Eight organ-based systems are separately assessed (outlined in Chapter 2, Section 2.4), according to the physician’s intention to treat. This information is entered into a computer and an alphabetic score is then obtained for each organ system. Patients attending the SLE clinic at the Centre for Rheumatology, University College London (see Chapter 2), are routinely assessed using the BILAG index.
**Results**

Importantly, an assessment of clinical disease activity using the BILAG index, in all the patients with SLE detailed in this dissertation, at the time of providing a sample of venous blood, has been recorded.

Previous studies have detailed associations of Hsps with active clinical subsets of SLE using the BILAG index. For example, elevated levels of Hsp 90 in patients with this disease have correlated with both active central nervous system and cardio-vascular/-respiratory disease and changes in Hsp 90 levels were observed to be sensitive to changes in haematological disease (Dhillon et al., 1993a). Levels of the cytokines IL-6 (Linker-Israeli et al., 1991) and IL-10 (Park et al., 1998) have been reported to be highest in patients with active disease. However, associations of these cytokines with specific organ/system involvement, using the BILAG index, have not been reported.

The objective of this chapter is to compare levels of Hsps, cytokines and antibodies to Hsps between active and inactive clinical subsets of SLE, using the BILAG index.
6.2 Heat Shock Proteins

Levels of Hsp 90 and Hsp 70 in PBMCs of patients with SLE, have been determined in Chapter 4. Levels of both Hsp 90 and Hsp 70 were assessed in patients graded by the BILAG index, by the methods outlined in Chapter 2.

Table 6.2.1 shows the number of patients with SLE, graded by the BILAG index, with either normal or elevated levels of Hsp 90. No significant correlations between levels of Hsp 90 (normal or elevated, as defined in Chapter 4) and disease activity grade were found, as determined by chi-square analyses (defined in Chapter 2). As shown in Figure 6.2.1, mean levels of Hsp 90 (-2SEM to +2SEM) were determined in patients graded by the BILAG index. No significant differences in mean Hsp 90 levels between grades were found, as determined by student's t-tests (also defined in Chapter 2) for equality of means.

Previous studies (Dhillon et al., 1993a) have associated elevated levels of Hsp 90 with overall active disease as defined by the UCH/Middlesex SLE disease activity index (Morrow et al., 1982; 1983). However, clinical disease activity data for the patients outlined in this chapter, as measured by this index, was not recorded.

Table 6.2.1 also shows the number of patients with SLE, graded by the BILAG index, with either normal or elevated levels of Hsp 70. Correlations between levels of Hsp 70 (normal or elevated, as defined in Chapter 4) and disease activity grade were not found to be significant, as determined by chi-square analyses. As shown in Figure 6.2.2, mean levels of Hsp 70 (-2SEM to +2SEM) were determined in patients graded by the BILAG index. Mean Hsp 70 levels were found to be significantly lower (p=0.004) in patients graded by BILAG A/B/C MUSK, compared with patients graded by BILAG D/E MUSK. No significant differences in mean Hsp 70 levels between other disease activity grades were found, as determined by student's t-tests for equality of means.
Results

Table 6.2.1  Number of Patients with SLE with Normal or Elevated Levels of Hsp 90 and Graded by BILAG Disease Activity Scores

<table>
<thead>
<tr>
<th>Hsp 90</th>
<th>BILAG Category</th>
<th>Hsp Level</th>
<th>Inactive Disease (Score D/E)</th>
<th>Active Disease (Score A/B/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GEN</td>
<td>Normal</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>SKIN</td>
<td>Normal</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CNS</td>
<td>Normal</td>
<td>52</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>MUSK</td>
<td>Normal</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>CV/RP</td>
<td>Normal</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>VASC</td>
<td>Normal</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>RENAL</td>
<td>Normal</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>HAEM</td>
<td>Normal</td>
<td>22</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elevated</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>
**Results**

(Table 6.2.1 continued)

**Hsp 70**

<table>
<thead>
<tr>
<th>BILAG Category</th>
<th>Hsp Level</th>
<th>Inactive Disease (Score D/E)</th>
<th>Active Disease (Score A/B/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>Normal</td>
<td>21</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>5</td>
<td>2 NS</td>
</tr>
<tr>
<td>SKIN</td>
<td>Normal</td>
<td>71</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>3</td>
<td>4 NS</td>
</tr>
<tr>
<td>CNS</td>
<td>Normal</td>
<td>72</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>2</td>
<td>4 NS</td>
</tr>
<tr>
<td>MUSK</td>
<td>Normal</td>
<td>43</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>5</td>
<td>2 NS</td>
</tr>
<tr>
<td>CV/RP</td>
<td>Normal</td>
<td>75</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Elevated</td>
<td>6</td>
<td>1 NS</td>
</tr>
<tr>
<td>VASC</td>
<td>Normal</td>
<td>60</td>
<td>35</td>
</tr>
<tr>
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Results

(Table 6.2.1 continued)

Key:

NS        not significant
n          number of patients
GEN       general non-specific disease
SKIN      mucocutaneous disease
CNS       central nervous system disease
MUSK      musculoskeletal disease
CVRP      cardiovascular and respiratory disease
VASC      vasculitis
RENAL     renal disease
HAEM      haematological disease
Figure 6.2.1 Mean Hsp 90 Levels (-2SEM to +2SEM) in Patients Graded by the BILAG Index

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Figure 6.2.2 Mean Hsp 70 Levels (-2SEM to +2SEM) in Patients Graded by the BILAG Index

p=0.004
Results

Legend to Figure 6.2.1 and Figure 6.2.2.

Bar charts showing mean levels (-2SEM to +2SEM) of Hsps in patients with SLE and graded by the BILAG index. Blue bars represent patients with inactive disease and grey bars represent patients with active disease, in individual BILAG organ/system categories. The P value (p=0.038) denotes the value for the statistical significance of the difference in mean Hsp 70 levels, between patients graded by active and inactive musculoskeletal disease, as determined by a student’s t-test (described in Chapter 2) for equality of means.

Key:

n number of patients
GEN general non-specific disease
SKIN mucocutaneous disease
CNS central nervous system disease
MUSK musculoskeletal disease
CVRP cardiovascular and respiratory disease
VASC vasculitis
RENAL renal disease
HAEM haematological disease
Results

Associations of Hsp 90 with Disease Activity in SLE in Patients who Overexpress Hsp 90

In view of the heterogeneity of factors likely to be involved in the pathogenesis of SLE (discussed in Chapter 1, Section 1.3), associations of Hsp 90 with clinical disease activity in SLE, may be most appropriately assessed in the subset of patients with elevated levels of Hsp 90. Thus, levels of Hsp 90 (in patients who were determined in Chapter 4 to overexpress Hsp 90) were compared in patients graded by the BILAG index.

As shown in Figure 6.2.3, mean levels of Hsp 90 (-2SEM to +2SEM) were higher (p=0.090) in patients graded by active (BILAG A/B/C) compared with inactive (BILAG D/E) general disease features (BILAG GEN), as determined by a students t-test for equality of means. No significant differences in mean levels of Hsp 90 (-2SEM to +2 SEM) were observed between other disease grades.
Figure 6.2.3 Mean Hsp 90 Levels (-2SEM to +2SEM) in Patients who Overexpress Hsp 90 and Graded by the BILAG Index

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Legend:
- A/B/C
- D/E
Results

Legend to Figure 6.2.3.

Bar chart showing mean levels (-2SEM to +2SEM) of Hsp 90 in patients with SLE who overexpress Hsp 90 and graded by the BILAG index. Blue bars represent patients with inactive disease and grey bars represent patients with active disease, in individual BILAG organ/system categories. The P value (p=0.090) denotes the value for the statistical significance of the difference in mean Hsp 90 levels, between patients graded by active and inactive general non-specific disease, as determined by a student’s t-test (described in Chapter 2) for equality of means.

Key:

n number of patients
GEN general non-specific disease
SKIN mucocutaneous disease
CNS central nervous system disease
MUSK musculoskeletal disease
CVRP cardiovascular and respiratory disease
VASC vasculitis
RENAL renal disease
HAEM haematological disease
6.3 Associations of HLA with Active Clinical Subsets in SLE

Previous studies (Dhillon et al., 1994) and the results presented in Chapter 4, suggest a genetic association with Hsp 90 overexpression in SLE. Thus it is conceivable, that any association between levels of Hsp 90 and active clinical subsets in SLE, may be due to factors genetically linked with Hsp 90 overexpression, but with roles in this disease independent of Hsp levels.

Table 6.3.1 shows the number of patients graded by the BILAG disease activity index, with respect to the presence or absence of individual HLA alleles/haplotypes. Significant associations of individual HLA alleles/haplotypes, as determined by chi-square analyses, are detailed in Table 6.3.2. The absence of HLA A1, B8, was associated with BILAG A/B/C VASC (p=0.014). The absence of HLA B8, DR3 was associated with BILAG A/B/C SKIN (p=0.018). No significant associations between HLA status and active (BILAG A/B/C) or inactive (BILAG D/E) general disease features (BILAG GEN) were observed.

Reports concerning correlations of individual HLA alleles/haplotypes with active clinical subsets of patients with SLE, have been conflicting. Indeed, the associations detailed above do not correspond to previous studies. Importantly, as discussed in Chapter 1, Section 1.3, such inconsistencies may derive from a complex involvement of ethnic background in patients with SLE, to any correlations involving individual HLA alleles/haplotypes with SLE. Interestingly however, in both a previous study (Dhillon et al., 1994) and in Chapter 4, the HLA DR3 negative haplotype has been associated with overexpression of Hsp 90 in patients with SLE. Moreover, Hsp 90 overexpression has been associated with active cardio-vascular/respiratory disease, in patients with SLE (Dhillon et al., 1993a).
**Table 6.3.1**  Associations of HLA with Active Clinical Subsets in SLE

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**SKIN**

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### Results

Table 6.3.1 (continued)

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### Results

#### Table 6.3.1 (continued)

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Table 6.3.1 (continued)

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</tr>
<tr>
<td>CVRP</td>
<td>cardiovascular and respiratory disease</td>
</tr>
<tr>
<td>VASC</td>
<td>vasculitis</td>
</tr>
<tr>
<td>RENAL</td>
<td>renal disease</td>
</tr>
<tr>
<td>HAEM</td>
<td>haematological disease</td>
</tr>
</tbody>
</table>
## Results

Table 6.3.2  Chi-Square Tests for the Correlation of HLA Alleles/Haplotypes With Active Clinical Subsets in SLE

### BILAG Category: SKIN

<table>
<thead>
<tr>
<th>Allele/Haplotype</th>
<th>Total</th>
<th>D/E</th>
<th>A/B/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>B8, DR3 present</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>B8, DR3 absent</td>
<td>61</td>
<td>40</td>
<td>101</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>70</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Chi-Square Significance

- Pearson: 0.018
- Likelihood Ratio: 0.003
- Linear by Linear Association: 0.018
- Fisher's Exact Test: One-tail 0.014, Two-tail 0.025

### BILAG Category: VASC

<table>
<thead>
<tr>
<th>Allele/Haplotype</th>
<th>Total</th>
<th>D/E</th>
<th>A/B/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1, B8 present</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A1, B8 absent</td>
<td>55</td>
<td>43</td>
<td>97</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>65</td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

Chi-Square Significance

- Pearson: 0.014
- Likelihood Ratio: 0.005
- Linear by Linear Association: 0.013
- Fisher's Exact Test: One-tail 0.011, Two-tail 0.015

**Key:**

- D/E: inactive disease
- A/B/C: active disease
- SKIN: mucocutaneous disease
- VASC: vasculitis
Results

6.4 Cytokines

As discussed in Chapter 1, Section 1.3, various studies have strongly supported a pathogenic role for the cytokines IL-6 and IL-10 in patients with SLE. Thus, using the BILAG index, an investigation of the associations of both cytokines with disease activity in patients with SLE, is outlined below.

Mean levels of IL-6 (± 2SEM) in patients graded by the BILAG index are shown in Figure 6.4.1. Higher mean IL-6 levels were found in patients graded by BILAG A/B/C HAEM, compared with those patients graded by BILAG D/E HAEM (p=0.037), as determined by a student’s t-test for equality of means. Associations of IL-6 levels in patients with SLE with other BILAG disease grades (Figure 6.4.1) or with BILAG GLOBAL disease activity scores (see Table 6.4.1), were not found to be significant.

Mean levels of IL-10 (± 2SEM) in patients graded by the BILAG disease activity scoring system are shown in Figure 6.4.2. Significantly higher mean IL-10 levels (p=0.020) were found in patients graded by BILAG D/E GEN, compared with those patients graded by BILAG A/B/C GEN, as determined by a student’s t-test for equality of means. Associations of IL-10 levels in patients with SLE with other BILAG disease grades, were not observed to be significant. Furthermore, as shown in Table 6.4.1, levels of IL-10 in patients with SLE were not found to correlate with BILAG GLOBAL disease activity scores.
Results

Table 6.4.1 Correlations of IL-6 and IL-10 with Global BILAG Disease Activity Scores in SLE

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>r</th>
<th>p</th>
<th>Test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All SLE</td>
<td>113</td>
<td>0.125</td>
<td>0.187</td>
<td>Spearman</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>113</td>
<td>0.084</td>
<td>0.204</td>
<td>Kendall</td>
<td>NS</td>
</tr>
<tr>
<td>Patients with Elevated Levels of Hsp 90</td>
<td>27</td>
<td>0.468</td>
<td><strong>0.014</strong></td>
<td>Spearman</td>
<td></td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All SLE</td>
<td>105</td>
<td>0.120</td>
<td>0.223</td>
<td>Spearman</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>0.079</td>
<td>0.252</td>
<td>Kendall</td>
<td>NS</td>
</tr>
<tr>
<td>Patients with Elevated Levels of Hsp 90</td>
<td>22</td>
<td>-0.308</td>
<td>0.163</td>
<td>Spearman</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>-0.218</td>
<td>0.170</td>
<td>Kendall</td>
<td>NS</td>
</tr>
</tbody>
</table>

**KEY:**

- **n**: number of patients
- **r**: correlation coefficient
- **p**: significance
- **NS**: not significant
Figure 6.4.1 Mean IL-6 Levels (-2SEM to +2SEM) in Patients Graded by the BILAG Index

<table>
<thead>
<tr>
<th>Condition</th>
<th>A/B/C</th>
<th>D/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>31</td>
<td>79</td>
</tr>
<tr>
<td>SKIN</td>
<td>70</td>
<td>40</td>
</tr>
<tr>
<td>CNS</td>
<td>93</td>
<td>17</td>
</tr>
<tr>
<td>MUSK</td>
<td>52</td>
<td>58</td>
</tr>
<tr>
<td>CVRP</td>
<td>93</td>
<td>17</td>
</tr>
<tr>
<td>VASC</td>
<td>66</td>
<td>44</td>
</tr>
<tr>
<td>RENAL</td>
<td>78</td>
<td>32</td>
</tr>
<tr>
<td>HAEM</td>
<td>41</td>
<td>69</td>
</tr>
</tbody>
</table>

p = 0.037
Figure 6.4.2 Mean IL-10 Levels (-2SEM to +2SEM) in Patients Graded by the BILAG Index

<table>
<thead>
<tr>
<th>Category</th>
<th>A/B/C</th>
<th>D/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>28</td>
<td>75</td>
</tr>
<tr>
<td>SKIN</td>
<td>64</td>
<td>39</td>
</tr>
<tr>
<td>CNS</td>
<td>87</td>
<td>16</td>
</tr>
<tr>
<td>MUSK</td>
<td>48</td>
<td>55</td>
</tr>
<tr>
<td>CVRP</td>
<td>87</td>
<td>16</td>
</tr>
<tr>
<td>VASC</td>
<td>62</td>
<td>41</td>
</tr>
<tr>
<td>RENAL</td>
<td>73</td>
<td>30</td>
</tr>
<tr>
<td>HAEM</td>
<td>38</td>
<td>65</td>
</tr>
</tbody>
</table>
Results

Legend to Figure 6.4.1 and Figure 6.4.2.

Bar chart showing mean levels (-2SEM to +2SEM) of cytokines in patients with SLE and graded by the BILAG index. Blue bars represent patients with inactive disease and grey bars represent patients with active disease, in individual BILAG organ/system categories. The P value (p=0.037) denotes the value for the statistical significance of the difference in mean IL-6 levels, between patients graded by active and inactive haematological disease, as determined by a student’s t-test (described in Chapter 2) for equality of means.

Key:

n number of patients
GEN general non-specific disease
SKIN mucocutaneous disease
CNS central nervous system disease
MUSK musculoskeletal disease
CVRP cardiovascular and respiratory disease
VASC vasculitis
RENAL renal disease
HAEM haematological disease
Results

Associations of IL-6 with Disease Activity in SLE in Patients who Overexpress Hsp 90

Any role for IL-6 in the pathogenesis of SLE must involve the activation of specific genes in target cells. Studies in Chapter 4 have suggested that a role for IL-6 in Hsp 90 overexpression in patients with SLE, has a genetic involvement. Thus, it is conceivable that the regulation of other genes by IL-6 in SLE, may have a similar genetic involvement.

In patients who were determined in Chapter 4 to overexpress Hsp 90, a comparison of IL-6 levels in these patients with BILAG GLOBAL disease activity scores (Table 6.4.1), showed significant correlations as determined by both Spearman (r=0.468, p=0.014) and Kendall (r=0.370, p=0.009) analyses.

Mean levels of IL-6 (-2SEM to +2SEM) in patients who were determined in Chapter 4 to overexpress Hsp 90, were graded by the BILAG index as shown in Figure 6.4.3. Associations of IL-6 levels in these patients with elevated Hsp 90, who were graded by cardio-vascular/-respiratory disease, showed significantly higher mean IL-6 levels in BILAG A/B/C patients compared with BILAG D/E patients (p=0.024). Interestingly, in a previous study of Hsp 90 associations with disease activity in SLE, overexpression of Hsp 90 in patients with this disease, was associated with active cardio-vascular/-respiratory disease (Dhillon et al., 1993a). A comparison of IL-6 levels in patients who overexpress Hsp 90 and who were graded by haematological disease activity, showed higher mean levels of IL-6 in BILAG A/B/C patients compared with BILAG D/E patients. However, an appreciably lower significance was observed in this analysis (p=0.860, see Figure 6.4.3) when compared to an analysis in patients without selection for Hsp 90 overexpression (p=0.037, see Figure 6.4.1). As shown in Figure 6.4.3, other disease associations with IL-6 levels in patients with SLE who overexpress Hsp 90, did not attain statistical significance.
Figure 6.4.3 Mean IL-6 Levels (-2SEM to +2SEM) in Patients who Overexpress Hsp 90 and Graded by the BILAG Index

$p = 0.024$

<table>
<thead>
<tr>
<th>System</th>
<th>A/B/C</th>
<th>D/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>SKIN</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>CNS</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>MUSK</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>CVRP</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>VASC</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>RENAL</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>HAEM</td>
<td>13</td>
<td>14</td>
</tr>
</tbody>
</table>
Results

Legend to Figure 6.4.3.

Bar chart showing mean levels (-2SEM to +2SEM) of IL-6 in patients with SLE who overexpress Hsp 90 and graded by the BILAG index. Blue bars represent patients with inactive disease and grey bars represent patients with active disease, in individual BILAG organ/system categories. The P value denotes the value for the statistical significance of the difference in mean IL-6 levels, between patients graded by active and inactive cardiovascular and respiratory disease (p=0.024) or haematological disease (p=0.860), as determined by a student’s t-test (described in Chapter 2) for equality of means.

Key:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>number of patients</td>
</tr>
<tr>
<td>GEN</td>
<td>general non-specific disease</td>
</tr>
<tr>
<td>SKIN</td>
<td>mucocutaneous disease</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system disease</td>
</tr>
<tr>
<td>MUSK</td>
<td>musculoskeletal disease</td>
</tr>
<tr>
<td>CVRP</td>
<td>cardiovascular and respiratory disease</td>
</tr>
<tr>
<td>VASC</td>
<td>vasculitis</td>
</tr>
<tr>
<td>RENAL</td>
<td>renal disease</td>
</tr>
<tr>
<td>HAEM</td>
<td>haematological disease</td>
</tr>
</tbody>
</table>
6.5 Antibodies to Heat Shock Proteins

As discussed in Chapter 1, Section 1.2, Hsp-specific responses have been observed in a number of autoimmune diseases including SLE and may have a role in the pathogenesis of these diseases.

In Chapter 5, a number of patients with SLE were determined to have raised levels of antibodies to Hsps (particularly Hsp 90). Table 6.5.1 shows the number of patients with SLE, graded by the BILAG index, with either normal or raised (defined in Chapter 5) levels of antibodies to Hsp 90. As shown in Table 6.5.2, raised levels of IgG antibodies to Hsp 90 were found to be significantly associated with BILAG A/B/C GEN patients (p=0.019), as determined by a chi-square analysis. Associations of IgG antibodies with other disease activity grades, were not found to be significant.

Table 6.5.1 also shows the number of patients with SLE, graded by BILAG disease activity scores, with either normal or raised (defined in Chapter 5) levels of IgM antibodies to Hsp 90. Associations of IgM antibodies to Hsp 90 with other disease activity grades, were not found to be significant.

Table 6.5.3 shows the number of patients with SLE, in each BILAG disease activity grade, with either normal or raised (defined in Chapter 5) levels of antibodies to Hsp 70. Analysis of individual organ/system involvement using the BILAG index, showed no significant disease associations with either levels of IgG or IgM antibodies to Hsp 70 in these patients, as determined by chi-square analyses.
### Results

Table 6.5.1  Number of Patients with SLE with Normal or Raised Levels of Antibodies to Hsp 90 in Inactive or Active BILAG Categories

**IgG Antibodies to Hsp 90**

<table>
<thead>
<tr>
<th>BILAG Category</th>
<th>Antibody Level</th>
<th>Inactive Disease (Score A/B/C)</th>
<th>Active Disease (Score D/E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>Normal</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>7</td>
<td>37  ( P=0.01 )</td>
</tr>
<tr>
<td>SKIN</td>
<td>Normal</td>
<td>41</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>23</td>
<td>21  ( \text{NS} )</td>
</tr>
<tr>
<td>CNS</td>
<td>Normal</td>
<td>48</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>37</td>
<td>7  ( \text{NS} )</td>
</tr>
<tr>
<td>MUSK</td>
<td>Normal</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>22</td>
<td>22  ( \text{NS} )</td>
</tr>
<tr>
<td>CV/RP</td>
<td>Normal</td>
<td>51</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>35</td>
<td>9  ( \text{NS} )</td>
</tr>
<tr>
<td>VASC</td>
<td>Normal</td>
<td>33</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>29</td>
<td>15  ( \text{NS} )</td>
</tr>
<tr>
<td>RENAL</td>
<td>Normal</td>
<td>41</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>32</td>
<td>12  ( \text{NS} )</td>
</tr>
<tr>
<td>HAEM</td>
<td>Normal</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>16</td>
<td>28  ( \text{NS} )</td>
</tr>
</tbody>
</table>
### IgM Antibodies to Hsp 90

<table>
<thead>
<tr>
<th>BILAG Category</th>
<th>Antibody Level</th>
<th>Inactive Disease (Score A/B/C)</th>
<th>Active Disease (Score D/E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>Normal</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>SKIN</td>
<td>Normal</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>CNS</td>
<td>Normal</td>
<td>52</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>MUSK</td>
<td>Normal</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>CV/RP</td>
<td>Normal</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>VASC</td>
<td>Normal</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>RENAL</td>
<td>Normal</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>HAEM</td>
<td>Normal</td>
<td>22</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>
Results

(Table 6.5.1 continued)

Key:

NS  not significant
n   number of patients
GEN general non-specific disease
SKIN mucocutaneous disease
CNS central nervous system disease
MUSK musculoskeletal disease
CVRP cardiovascular and respiratory disease
VASC vasculitis
RENAL renal disease
HAEM haematological disease
### Results

Table 6.5.2 Chi-Square Test for the Correlation of Active BILAG General Disease with Raised IgG Antibodies to Hsp 90 in SLE

<table>
<thead>
<tr>
<th>Antibody Level</th>
<th>Inactive Disease</th>
<th>Active Disease</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>23</td>
<td>35</td>
<td>58</td>
</tr>
<tr>
<td>Raised</td>
<td>7</td>
<td>37</td>
<td>44</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>30</strong></td>
<td><strong>72</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chi-Square</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearson</td>
<td>0.009</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>0.008</td>
</tr>
<tr>
<td>Linear by Linear Association</td>
<td>0.009</td>
</tr>
<tr>
<td>Fisher's Exact Test</td>
<td>0.015 One-tail</td>
</tr>
<tr>
<td></td>
<td>0.008 Two-tail</td>
</tr>
</tbody>
</table>
Table 6.5.3  Number of Patients with SLE with Normal or Raised Levels of Antibodies to Hsp 70 in Inactive or Active BILAG Categories

**IgG Antibodies to Hsp 70**

<table>
<thead>
<tr>
<th>BILAG Category</th>
<th>Antibody Level</th>
<th>Inactive Disease (Score A/B/C)</th>
<th>Active Disease (Score D/E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>Normal</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>SKIN</td>
<td>Normal</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>CNS</td>
<td>Normal</td>
<td>52</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>MUSK</td>
<td>Normal</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>CV/RP</td>
<td>Normal</td>
<td>51</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>VASC</td>
<td>Normal</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>RENAL</td>
<td>Normal</td>
<td>39</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>HAEM</td>
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<td>22</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>
Results

(Table 6.5.3 continued)

**IgM Antibodies to Hsp 70**

<table>
<thead>
<tr>
<th>BILAG Category</th>
<th>Antibody Level</th>
<th>Inactive Disease (Score A/B/C)</th>
<th>Active Disease (Score D/E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEN</td>
<td>Normal</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Raised</td>
<td>6</td>
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<tr>
<td>SKIN</td>
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</tr>
<tr>
<td></td>
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<tr>
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<td>Normal</td>
<td>52</td>
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</tr>
<tr>
<td></td>
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<td>25</td>
<td>5</td>
</tr>
<tr>
<td>MUSK</td>
<td>Normal</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td></td>
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<td>12</td>
<td>18</td>
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<tr>
<td>CV/RP</td>
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<td>51</td>
<td>10</td>
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<td></td>
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<td>26</td>
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<tr>
<td>VASC</td>
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NS: Not Significant
**Results**

*(Table 6.5.3 continued)*

**Key:**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>n</td>
<td>number of patients</td>
</tr>
<tr>
<td>GEN</td>
<td>general non-specific disease</td>
</tr>
<tr>
<td>SKIN</td>
<td>mucocutaneous disease</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system disease</td>
</tr>
<tr>
<td>MUSK</td>
<td>musculoskeletal disease</td>
</tr>
<tr>
<td>CVRP</td>
<td>cardiovascular and respiratory disease</td>
</tr>
<tr>
<td>VASC</td>
<td>vasculitis</td>
</tr>
<tr>
<td>RENAL</td>
<td>renal disease</td>
</tr>
<tr>
<td>HAEM</td>
<td>haematological disease</td>
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</table>
6.6 Discussion

A role for Hsp 90 in the pathogenesis of SLE has been discussed in Chapter 1, Section 1.4. These studies have suggested that overexpression of Hsp 90, which occurs in a subset of patients with SLE, may lead to the development of antibodies to Hsp 90 in these patients, with possible pathological consequences. In agreement with this hypothesis, elevated levels of Hsp 90 (Faulds et al., 1994) and antibodies to Hsp 90 (Faulds et al., 1995) have been reported in lupus-like MRL-lpr/lpr mice and are events which precede the onset of disease. An understanding of the regulation of Hsp 90 in SLE is clearly of interest.

Previous studies (see Chapter 1, Section 1.3) have also suggested pathogenic roles for elevated levels of IL-6 and IL-10 in SLE. Of particular relevance to this dissertation, are studies which have supported a role for IL-6 (Stephanou et al., 1997), (Stephanou et al., 1998c) and IL-10 (Ripley et al., 1999), in the overexpression of Hsp 90 in SLE. Importantly, studies in Chapter 4 have shown that overexpression of Hsp 90 in SLE correlated with elevated levels of IL-6 and not IL-10, in patients with this disease.

However, the relative contribution of cytokines, Hsps and antibodies to Hsps, to disease activity in SLE, has remained unclear. In this chapter, a comparison of these factors between active and inactive clinical subsets of SLE, using the BILAG index for the measurement of clinical disease activity, has been undertaken.

Correlations between levels of Hsp 90 or Hsp 70 and disease activity in SLE were not found to be significant. These observations are not in agreement with the study of Dhillon et al., (1993a), which reported an association between elevated levels of Hsp 90 and active cardio-vascular/respiratory disease. However, in this chapter, associations of clinical subsets of SLE with the presence or absence of individual/combined HLA types, showed an association between the HLA DR3 negative haplotype and active cardio-vascular/respiratory disease. A common link between these two observations is that the HLA DR3 negative haplotype in SLE has been associated with Hsp 90 overexpression, both in the study of Dhillon et al., (1994) and in the results presented in Chapter 4. Thus, it is conceivable that the reported association of active cardio-vascular/respiratory disease with elevated levels
Results

of Hsp 90 in the study of Dhillon et al., (1993a), may have been influenced by factors other than Hsp 90 itself.

In this chapter, levels of IL-6 and IL-10 were compared both with active clinical subsets of and overall disease activity in, patients with SLE. An association between IL-6 and active haematological disease (BILAG A/B/C HAEM) was observed and was not paralleled by significant associations of IL-6 with other clinical profiles. Interestingly, in a previous study (Dhillon et al., 1993a), increasing levels of Hsp 90 in patients with SLE over time, were found to be associated with the development of haematological disease. Since the results in Chapter 4 support a role for IL-6 in the regulation of Hsp 90 expression in SLE, it is possible that the observation of Dhillon et al., (1993a) relates to an involvement of IL-6 in the development of haematological disease.

Levels of IL-6 were not observed to correlate with GLOBAL BILAG disease activity scores. In a previous study, using the SLEDAI and SLAM indexes (defined in Chapter 1, Section 1.3), a lack of correlation between levels of IL-6 and clinical profiles and between levels of IL-6 and overall disease activity, was reported (Grondal et al., 2000). However, in other studies, which measured levels of IL-6 both before and after disease exacerbations (Spronk et al., 1992), or at various stages of disease activity (Waszczykowska et al., 1999), levels of IL-6 correlated well with overall disease activity. It is possible therefore, that any relationship between IL-6 and disease activity in SLE may be most appropriately assessed by an assessment of changes in these variables in the same patients over time.

The absence of a correlation between levels of IL-10 and active clinical subsets in SLE or overall disease activity scores (BILAG GLOBAL) is in agreement with the study of Grondal et al., (2000). However, serum IL-10 levels have been shown to correlate with overall disease activity, when serial measurements are taken in the same patients over time (Waszczykowska et al., 1999). Thus, as for IL-6, any relationship between IL-10 and disease activity, may be most appropriately assessed in patients following significant changes in either variable over time.

Interestingly, studies in this chapter have shown a significant correlation between levels of IL-6 and overall disease activity scores (BILAG GLOBAL), in patients who overexpressed Hsp 90. One possible explanation for this, in agreement
Results

with the studies in Chapter 4, is that Hsp 90 overexpression may be a marker for recent and significant elevation of IL-6 levels in these patients. Moreover, any relationship between IL-6 and disease activity, must be explainable in terms of IL-6 activation of its target genes. Thus, since a correlation between IL-6 and Hsp 90 is enhanced in patients who overexpress Hsp 90, any involvement of IL-6 in disease activity may also be enhanced in this subset of patients.

A comparison of mean levels of IL-6 in patients who overexpress Hsp 90 and graded by the BILAG index, showed significantly higher levels of IL-6 in patients with active compared with inactive cardio-vascular/respiratory disease. This observation is of particular significance since, as discussed earlier, previous studies (Dhillon et al., 1993a) and the results presented in this dissertation, suggest an association of active cardio-vascular/respiratory disease with factors associated with Hsp 90 overexpression and/or with HLA DR3 negativity in this disease. Thus, the observed associations in SLE of elevated levels of Hsp 90 (Dhillon et al., 1993) and of HLA DR3 negativity, with active cardio-vascular/respiratory disease, may have been most strongly influenced by elevated levels of IL-6 in a subset of patients with SLE.

Associations of raised levels of antibodies to Hsp 90 with active clinical subsets of patients with SLE, were not found to be significant for analyses involving any one organ/system. Interestingly however, a significant correlation between raised IgG antibodies to Hsp 90 and active general disease features (BILAG A/B/C GEN), was found.

Previous studies have correlated general disease features with severity of illness (Krupp et al., 1990), (Wysenbeek et al., 1993) in patients with SLE. Moreover, in another study, patients with active general disease features were associated with severe tissue inflammation (Amit et al., 1999). Thus, a significant correlation between the presence of raised antibodies to Hsp 90 and active general disease features (BILAG A/B/C GEN) in patients with SLE, would suggest a link between these antibodies and active disease. Furthermore, such a relationship may be most relevant to the presence of severe inflammation, rather than the prevalence of organ/system involvement.
Results

This observation is of particular interest, since antibodies to Hsps in autoimmune diseases, have been suggested to both promote and sustain autoaggression at sites of tissue inflammation (see Chapter 1, Section 1.2). Such a role, if relevant to antibodies to Hsp 90 in the pathogenesis of SLE, would therefore involve an enhancement of and occur secondary to, the presence of active disease in one or several organ/systems. A significant correlation between raised levels of IgG antibodies to Hsp 90 and active general disease features (BILAG A/B/C GEN), in the absence of significant correlations of raised levels of these antibodies with other active clinical subsets of SLE, is consistent with a role for these antibodies in sustaining and enhancing autoaggression and tissue inflammation, in a non-organ/system-specific manner. Arguably, the induction of antibodies to Hsp 90, may occur as part of, but not be involved with, active disease. However, the complexity of aetiopathogenic factors in SLE (discussed in Chapter 1, Section 1.3), would suggest this to be unlikely.

Taken together, these results suggest that any involvement of Hsp 90 in the pathogenesis of SLE, is most likely to be mediated by Hsp 90 overexpression and the development of antibodies to Hsp 90 in SLE, which leads to active general disease features which suggestively are a consequence of enhanced tissue inflammation.
Chapter 7

General Discussion

This dissertation describes an investigation of the role of cytokines in the expression of heat shock proteins (Hsps) in systemic lupus erythematosus (SLE). Studies in all the previous results chapters presented in this dissertation, are discussed together in this chapter.

Previous studies have established important cellular roles for Hsps under various stressful conditions and these appear to be an extension of their role in unstressed cells. Thus, some Hsps are expressed constitutively at high levels in cells and have roles in cell growth and development (Bond and Schlesinger, 1987), while other Hsps are present at much lower levels in cells and their synthesis is enhanced in response to stressful conditions (Morimoto, 1993). Although the mechanisms underlying inducible Hsp gene expression under stressful conditions have been intensively studied, the regulation of Hsps in unstressed cells has remained less well characterised. Such studies are of importance since Hsps have been observed to be differentially expressed following viral infection (La Thangue and Latchman, 1988), in different tumour cell lines (Ferrarini et al., 1992) and in specific disease states (Jameel et al., 1992).

As outlined in Chapter 1, Section 1.4, previous studies (Norton et al., 1989), (Dhillon et al., 1993a) have reported the differential expression of Hsps in SLE and are reviewed in Stephanou et al., (1998c). Thus, levels of Hsp 90 are elevated in PBMCs of up to 30% of patients with SLE, compared to normal control subjects. Moreover, this elevation occurs in the absence of elevation of other Hsps, including Hsp 73 and Hsp 60 (Dhillon et al., 1993a) and ubiquitin (Twomey et al., 1992). Thus, these studies suggest that overexpression of Hsp 90 in SLE, occurs via distinct regulatory mechanisms and not as a general response to stress, which would result in elevated levels of a wide range of Hsps.
Discussion

Recent studies have shown that the cytokine interleukin-6 (IL-6) enhances the expression of Hsp 90 in different cells in vitro (Stephanou et al., 1997) and that such elevation of Hsp 90 levels is also observed in vivo in transgenic mice overexpressing IL-6 (Stephanou et al., 1998a). Activation of the Hsp 90 promoter is mediated by the IL-6-induced activation of the transcription factors NF-IL6 (nuclear factor for IL-6) and STAT-3 (signal transducer and activator of transcription-3) and involves a short specific region of the promoter which contains binding sites for these factors (Stephanou et al., 1997), (Stephanou et al., 1998b). Thus, in view of the known role of STAT proteins in signalling pathways mediated by the cytokine interleukin-10 (IL-10), an investigation of the effect of IL-10 on Hsp 90 gene expression has been presented in Chapter 3.

These studies have demonstrated that IL-10 can also enhance Hsp 90 levels in different cells in vitro and that this effect is mediated via the same short specific region of the Hsp 90 promoter which is targetted by IL-6 (Ripley et al., 1999). Moreover, since activation of the Hsp 90α and -β gene promoters was enhanced by cotransfection of a STAT-3 expression vector, it is likely that the observed effect of IL-10 on Hsp 90 gene expression, is mediated via the IL-10-induced activation of STAT-3. Hence, the effect of IL-10 on Hsp 90 levels in vitro is comparable to that of IL-6 and is likely to reflect the similarity of IL-10 signalling mechanisms to that of IL-6, through the activation of STAT-3 (Lai et al., 1996). Furthermore, since murine studies have demonstrated that elevation of Hsp 90 levels by IL-6 can occur in the absence of NF-IL-6 (Stephanou et al., 1998a), which is activated by IL-6 and not IL-10 (Finbloom and Winestock, 1995), these observations suggest a shared pathway for IL-10 and IL-6 in stimulation of Hsp 90 in vivo.

Important cellular roles for Hsps in the immune system have been detailed in Chapter 1, Section 1.2 and these appear to be an extension of their known roles as molecular chaperones. Hence, any role for cytokines in the regulation of Hsp gene expression in vivo, may reflect the importance of Hsp function in such processes as antigen presentation and the production of immunoglobulins, during an immune response. Moreover, since IL-6 (Linker-Israeli et al., 1991) and IL-10 (Park et al., 1998) have been reported to be elevated in patients with SLE, these observations may
have important implications for the mechanisms of Hsp 90 overexpression in this disease.

Studies in Chapter 4 of this dissertation, have shown a differential pattern of expression of Hsps in patients with SLE. Thus, in agreement with a previous study (Dhillon et al., 1993a), Hsp 90 was observed to be overexpressed in PBMCs in a subset of patients with SLE and not in patients with rheumatoid arthritis, compared to control normal individuals. This elevation was specific to Hsp 90 and was not paralleled by elevation of Hsp 70 in the same patients with SLE. An investigation of the relative effect of the cytokines IL-6 and IL-10, on the overexpression of Hsp 90 in SLE, has also been outlined in Chapter 4. Levels of serum IL-6 were found to correlate with levels of Hsp 90 but not Hsp 70, in PBMCs of individual patients with SLE. In contrast, levels of serum IL-10 were not found to correlate with levels of Hsp 90 nor with levels of Hsp 70. Thus, these results suggest a role for IL-6 in the overexpression of Hsp 90 in patients with SLE and is in agreement with a previous study in transgenic mice, where elevated levels of IL-6 resulted in enhanced Hsp 90 protein levels (Stephanou et al., 1998a).

Levels of IL-6 were observed to be significantly higher in patients who overexpressed Hsp 90 compared to those patients with normal levels. Interestingly, this elevation of IL-6 was observed to be more significant than the observed elevation of Hsp 90 in patients with elevated levels of IL-6 compared to those with normal levels of IL-6 (see Figure 4.4.5 and Figure 4.4.6). Moreover, the observed correlation between IL-6 and Hsp 90 was stronger in patients who overexpressed Hsp 90 compared to an analysis in which all patients with SLE were studied (see Figure 4.4.1 and Figure 4.4.7). Hence, these observations suggest that an effect of IL-6 on Hsp 90 elevation in SLE, is strongest in a particular subset of patients with this disease. This may explain why elevation of Hsp 90 occurs in SLE and is not observed in other diseases such as rheumatoid arthritis (Dhillon et al., 1993a), which also features elevated levels of IL-6 (Stuart et al., 1995).

Studies presented in Chapter 4 have shown that overexpression of Hsp 90 in patients with SLE, was most likely to occur in patients who had an HLA DR3-negative haplotype and this observation is in agreement with the study of (Dhillon et al., 1994). As has been discussed in Chapter 4, the gene encoding Hsp 90β has been
Discussion

located on human chromosome 6 in the MHC region. Hence, it is conceivable that polymorphisms exist within the Hsp 90β promoter, which mediate the overexpression of Hsp 90 in response to elevated levels of IL-6, in a genetic subset of patients with SLE. However, the true significance of the association between patients lacking the HLA DR3 allele and Hsp 90 overexpression in SLE, requires the study of a greater number of patients in various ethnic groups, than those used in either this dissertation or in the study of Dhillon et al., (1994). This would permit an analysis of any effect of population ethnic bias/HLA haplotype frequencies on this reported association, which was not attainable in either study.

Raised levels of antibodies to Hsp 90 have been reported in patients with SLE of both adult (Conroy et al., 1994) and childhood-onset (Conroy et al., 1996). However, the mechanisms leading to this event have remained unclear. Previous studies have reported an association of the HLA DR3-negative haplotype with the presence of antibodies to Hsp 90 in patients with SLE (Conroy et al., 1994). As discussed earlier, the HLA DR3-negative haplotype in patients with SLE, has been associated with Hsp 90 overexpression both in a previous study (Dhillon et al., 1994) and in Chapter 4. Thus, these observations have led to a hypothesis that overexpression of Hsp 90 in SLE, results in the development of antibodies to Hsp 90 in the same patients (Stephanou et al., 1998c).

Studies in Chapter 5 have shown an association between overexpression of Hsp 90 and the presence of serum IgG antibodies to Hsp 90, in the same patients. Since levels of IL-6 were not observed to correlate with levels of IgG or IgM antibodies to Hsp 90, these observations suggest that the development of antibodies to Hsp 90 in SLE, occur as a result of Hsp 90 overexpression and not as a general B cell stimulatory effect mediated directly by IL-6. However, a more precise time-course experiment, to explore the link between IL-6, Hsp 90 and antibodies to Hsp 90, has not been undertaken. Such a study would take into account other factors, such as the serum half-life of human IgG antibody molecules being approximately 14 days (Stites et al., 1997).

As discussed in Chapter 1, Section 1.2, the development of autoantibodies within the immune system may have pathological consequences. Thus, in the normal immune system, such events are appropriately down-regulated and short-lived. Any
mechanism responsible for the development of antibodies to Hsp 90, is likely to require both the altered accessibility of Hsp 90 to cells of the immune system and other factors which sustain antibody production. Previous studies have reported the increased surface expression of Hsp 90 in PBMCs of patients with SLE (Erkeller-Yuksel et al., 1992). Moreover, elevated levels of IL-10, which feature in patients with SLE (Park et al., 1998), have been suggested to promote B cell hyperactivity and the increased production of antibodies in this disease (Rousset et al., 1992), (Kirou and Crow, 1999).

Taken together, these observations suggest that overexpression of Hsp 90 and its surface expression (Erkeller-Yuksel et al., 1992), results in the production of antibodies to Hsp 90 in patients with SLE.

Previous studies have suggested a pathogenic role for Hsp 90 in SLE. For example, elevated levels of Hsp 90 have been associated with overall active disease in patients with SLE (Dhillon et al., 1993a), using the UCH/Middlesex SLE disease activity index (Morrow et al., 1982; 1983). Furthermore, in the same study, using the BILAG disease activity index (defined in Chapter 2 and Appendix A), levels of Hsp 90 were observed to be higher in patients with active compared with inactive, cardiovascular/respiratory disease. Murine studies also support a role for Hsp 90 in the pathogenesis of SLE. Thus, in autoimmune MRL/lpr-lpr mice, which develops a lupus-like disease (see Chapter 1, Section 1.3), elevation of Hsp 90 protein levels and the development of antibodies to Hsp 90, are events which precede the onset of disease (Faulds et al., 1994).

A previously held hypothesis for the role of Hsp 90 in SLE, has been a process in which elevation of Hsp 90 in PBMCs results in the development of antibodies to Hsp 90, with pathological consequences for patients with this disease. In support of this hypothesis, previous studies (outlined in Chapter 1, Section 1.2) have correlated Hsp-specific antibody responses with active tissue disease. However, in patients with SLE, the relative involvement of Hsp 90 overexpression and antibodies to Hsp 90 in the pathogenesis this disease, has remained unclear. Thus, levels of these factors in the same patients, have been compared between active and inactive clinical subsets of SLE using the BILAG index. It should be noted however, that some statistical analyses of disease associations, involved small patient numbers in
Discussion

individual clinical subsets. This is particularly evident in comparisons of mean Hsp90 (see Figure 6.2.3), IL-6 (see Figure 6.4.3) levels, in patients who overexpress Hsp 90 and graded by the BILAG index. Hence, care should be taken against over-interpreting the significance of disease associations presented in this dissertation, where statistical analyses involved small patient numbers.

Studies presented in Chapter 6, have shown a correlation between levels of Hsp 90 and the presence of active general disease features of SLE. Furthermore, an enhanced correlation is observed in a comparison of raised levels of IgG antibodies to Hsp 90 and active general disease features. Associations of other clinical subsets of SLE using the BILAG index, with either levels of Hsp 90 or raised levels of antibodies to Hsp 90, did not prove to be statistically significant. Moreover, since elevated levels of IL-6 were not associated with active general disease features in patients with SLE, these observations support a role for Hsp 90 in the pathogenesis of SLE, which is mediated most strongly by the development of antibodies to Hsp 90.

As discussed in Chapter 1, Section 1.2, antibodies to Hsps have been localised in regions of active tissue disease and their presence has correlated with the degree of overexpression of Hsps at these sites. These observations underline a current theory of the pathogenic involvement of Hsp-specific antibody responses in autoimmune disease. This is a process in which the development of antibodies to Hsps, in patients with active autoimmune pathology, results in binding of antibody to Hsps localised at regions of active tissue disease, leading to enhanced inflammation. Such a process is therefore non-organ-specific, but rather, specific to the presence of active tissue disease. Importantly therefore, the observation in Chapter 6 of an association between antibodies to Hsp 90 and active general disease features of SLE, in the absence of a correlation with organ-specific disease, is in agreement with this hypothesis. Moreover, this conclusion is strengthened by the reported association between active general disease features and enhanced tissue inflammation (Amit et al., 1999). A hypothetical model of the pathogenic role of Hsp 90 in SLE is provided in Figure 7.1.
Discussion

Concluding Comments

The studies presented in this dissertation have provided evidence of a role for the cytokine IL-6 in the overexpression of Hsp 90 in a subset of patients with SLE. Furthermore, a link between the overexpression of Hsp 90 and the development of antibodies to Hsp 90 in the same patients, leading to active disease, has also been established.
Figure 7.1 illustrates a hypothetical model for a pathogenic role of Hsp 90 in patients with SLE.
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Appendix A

The BILAG Index


General Non-Specific Manifestations

1. Pyrexia.
2. Unintentional Weight Loss.
3. Lymphadenopathy.
4. Fatigue/malaise/weakness.
5. Anorexia/nausea/vomiting.

Score A pyrexia + two other categories.
Score B pyrexia or two other categories.
Score C any other one category.
Score D no current involvement.
Score E no current/never any involvement.

Mucocutaneous Disease

Score A- any one of the following categories:

1. Severe maculopapular, discoid or bullous eruption and/or extensive scarring or causing disability.
2. Angio-oedema.
3. Extensive mucosal ulceration.
Appendix A

Score **B**- any one of the following categories:

1. Malar erythema.
2. Mild maculopapular eruption.
3. Panniculitis.
4. Localised active discoid lesions including lupus profundus.
5. Severe active alopecia.
7. Perniotic skin lesions.

Score **C**- any one of the following categories:

1. Peri-ungual erythema.
2. Swollen fingers.
3. Sclerodactyly.
5. Telangectasia.
7. Small mucosal ulceration.

Score **D**- no current involvement.

Score **E**- no current/never any involvement.

**Central Nervous System Involvement**

**First Assessment.**

Score **A**- acute/progressive/recurring any one of the following categories:

1. Impaired consciousness.
2. Psychosis/delerium/confusional state.
Appendix A

5. Aseptic meningitis.
7. Ascending or transverse myelitis.
8. Peripheral or cranial neuropathy.
10. Cerebellar ataxia.

Score B- any one of the following categories:

1. Headache (severe unremitting).
2. Organic depressive illness.
3. Chronic brain syndrome including pseudotumour cerebri.
4. Disc swelling or cytoid bodies.

Score C- episodic migrainous headaches.

Score D- no current involvement.

Score E- no current/never any involvement.

Subsequent Assessments:

Score A- as for the first assessment (category A1-10), scored “worse” or “new”.

Score B- as for the first assessment (category B1-4) scored “worse” or “new” in the last month; or category A1-3 scored “same” or “improving” in the last month.

Score C- as for the first assessment (category C1); or category A4-10 scored “same” or “improving” in the last month.
Appendix A

Score D- no current involvement.

Score E- no current/never any involvement.

Musculoskeletal Disease

Score A- one/both of the following categories:

1. Definite myositis.
2. Severe polyarthritis with loss of function (not responsive to steroids <10 mg/day, antimalarials, non-steroidal therapy).

Score B- any one of the following categories:

1. Arthritis (definite synovitis).
2. Tendonitis.

Score C- any one of the following categories:

1. Arthralgia.
3. Tendon contractures and fixed deformity.
4. Aseptic necrosis.
5. Mild chronic myositis.

Score D- no current involvement.

Score E- no current/never any involvement.
Appendix A

Cardiovascular and Respiratory Disease

Score A- cardiac failure or symptomatic effusion + any two of the following categories; or any four of the following categories:

1. Pleuropericardial pain.
2. Dyspnoea.
3. Friction rub.
4. Progressive chest X-ray changes (lung fields).
5. Progressive chest X-ray changes (heart size).
6. ECG evidence of pericarditis or myocarditis.
7. Cardiac erythema including tachycardia: >100 in the absence of fever.
8. Deteriorating lung function: <20% of expected or > 20% fall.

Score B- any two categories from A1-9.

Score C- mild intermittent chest pain; or any category from A1-9.

Score D- no current involvement.

Score E- no current/never any involvement.

Vasculitis

Score A- any one of the following categories:

1. Major cutaneous vasculitis (including ulcers) + infarction in the past month.
2. Major abdominal crisis due to vasculitis.
3. Recurrent thrombo-embolism (excluding stroke).
Appendix A

Score B- any one of the following categories:

1. minor cutaneous vasculitis (nailfold/digital vasculitis, purpura, urticaria).
2. Superficial phlebitis.
3. Thromboembolism (excluding stroke), first episode.

Score C- any one of the following categories:

1. Raynaud's phenomenon.
2. Livedo reticularis.

Score D- no current involvement.

Score E- no current/never any involvement.

Renal Disease

First Assessment.

Score A- two or more of the following categories, including 1, 4 or 5:

1. Proteinuria (>1 g/24 hr or 3+/4+ dipstick).
2. Accelerated hypertension.
3. Creatinine clearance <50 ml/mn.
4. Active urinary sediment (on a non-centrifuged specimen): pyuria (>5wc/hpf); haematuria (>5rbc/hpf); or red cell casts in the absence of infection.
5. Histological evidence of active nephritis within the last three months.

Score B- any one of the following categories:

1. One category from A1-5.
2. Urinary dipstick 2+ or more.
Appendix A

3. 24 hr urinary protein >0.5 g <1 g.

Score C- any one of the following categories:

1. Urinary dipstick 1+.
2. B.P. >140/90 (5th phase).
3. Creatinine >130 mmol/l.

Score D- no current involvement.

Score E- no current/never any involvement.

Subsequent Assessments.

Score A- two or more of the following categories, including 1, 4 or 5:

1. Proteinuria (urinary dipstick increased by two or more levels); or 24 hr urinary protein rising from >0.2 g to >1 g; or 24 hr urinary protein rising from > 1 g by 100% or more; or newly documented proteinuria of > 1g.
2. Accelerated hypertension.
3. Deteriorating renal function: plasma creatinine >130 mmol/l and having risen to >130% of its previous value; or creatinine clearance having fallen to <67% of its previous value; or creatinine clearance <50 ml/mn and last time was >50 ml/mn, or was not measured.
4. Active urinary sediment (defined above).
5. Histological evidence of active nephritis (defined above).

Score B- any one of the following categories:

1. one category from A1-5.
2. Moderate proteinuria: urinary dipstick 2+ or more; or 24 hr urinary protein rising from >1 g by >50% but <100%.
Appendix A

3. Moderate decline in renal function (plasma creatinine >130 mmol/l or having risen to 115% of previous value.

Score C- any one of the following categories:

1. 24 hr urinary protein >0.25 g.
2. Urinary dipstick 1+ or more.
3. Rising B.P. (systolic rise of >30 mmHg or diastolic rise of >15 mmHg, provided recorded values are > 140/90).

Score D- no current involvement.

Score E- no current/never any involvement.

Haematological Disease

Score A- any one of the following categories:

1. Wcc <1000x10^9/l.
2. Platelet count <25x10^9/l.
3. Hb <8 g/dl.

Score B- any one of the following categories:

1. Wcc <2500x10^9/l.
2. Platelet count <100x10^9/l.
3. Hb <11 g/dl.
4. Evidence of active haemolysis (raised bilirubin +/- reticulocyte count and positive Coombs’ test).