ANTIBODIES TO HEAT SHOCK PROTEINS IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS AND IN PATIENTS WITH BREAST CANCER

by

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ABSTRACT

Elevated levels of heat shock protein 90 (hsp90) have been detected in the peripheral blood mononuclear cells (PBMCs) of approximately 25% of patients with systemic lupus erythematosus (SLE) compared to healthy controls. In contrast there is no increase in the expression of the constitutive hsp73, hsp60 or in the rate of transcription of the ubiquitin gene in SLE patients. Hence, among lupus patients, elevation of hsp90 is restricted to a subset of patients and is not a general response to cellular stress.

Although not normally expressed on the cell surface, hsp90 has been found on the surface of PBMCs in approximately 20% of patients with SLE. Thus the increased levels of hsp90 in some of the SLE patients may result in a proportion of the protein, or some component of it, becoming localised to the cell surface. The hsp90 on the cell surface could thus become the target of the immune system leading to the production of autoantibodies to hsp90.

To investigate this hypothesis a suitable method for detecting these antibodies in sera was devised. Previous methodology used a complex Western blot system and resulted in conflicting data. An ELISA was established to detect antibodies to native hsp90 in the sera from patients with SLE, other autoimmune diseases and healthy controls. IgM and IgG anti-hsp90 antibodies were found in 35% and 26% of SLE patients respectively. An ELISA was established to investigate whether these antibodies were part of a generalised anti-hsp response or specific to hsp90. In contrast less than 10% of patients had elevated levels of antibodies of either isotype to hsp70. This observation was extended to investigate antibodies to hsps to children with SLE, chronic arthritis, dermatomyositis and non-autoimmune controls.

Increased hsp expression has been observed in patients with malignant cancers with variable prognostic associations. Elevated hsp90 has been shown to correlate with poor prognosis in patients with breast cancer. Sera from 126 patients with breast cancer were tested for antibodies to hsp90 using the ELISA described above. Elevated levels of anti-hsp90 antibodies were detected in 38% of patients with malignant but not benign breast cancer and correlations with clinical parameters were investigated.
Acknowledgements

I am very grateful to Professor David Latchman for his guidance and supervision throughout the course of this thesis. I would also like to thank Professor David Isenberg for his help, particularly in obtaining the autoimmune disease sera, as well as making facilities in his laboratory available.

I would also like to thank Gary Faulds for many long discussions and general support throughout this PhD as well as Dr. Breda Twomey, Dr. Peter Sasieni, Dr. Andrea Watson, Dr. Warren Williams and John Esteridge for their encouragement, technical help and constructive criticism. I am grateful to Dr. Lori Tucker for providing the juvenile autoimmune sera and Dr. Luqmani and Gill Brünstrom for the breast cancer sera. I am particularly grateful to Dr. Lynn Rose for reading and constructively criticising this thesis and to Ann Maitland for proof-reading a large proportion of this work. I would especially like to thank Stephen Gibson for his moral support, encouragement as well as statistical advice.

Finally I would like to acknowledge financial support from the Medical Research Council.
Dedication

To my parents, Gerald and Sheila Conroy, as thanks for their support and encouragement over the years.
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<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium per sulphate</td>
</tr>
<tr>
<td>ARA</td>
<td>American and Rheumatism Association</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BIC</td>
<td>Carbonate-bicarbonate</td>
</tr>
<tr>
<td>BILAG</td>
<td>British Isles Lupus Assessment Group</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3 determining region</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous disease</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentary rate</td>
</tr>
<tr>
<td>ETDA</td>
<td>Ethylene diamino tetra acetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</td>
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<tr>
<td>GM</td>
<td>Growth medium</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GS</td>
<td>Goat sera</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine-aminopterin-thymidine</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen</td>
</tr>
<tr>
<td>HS</td>
<td>Heat shock</td>
</tr>
<tr>
<td>HSE</td>
<td>Heat shock element</td>
</tr>
<tr>
<td>HSF</td>
<td>Heat Shock Factor</td>
</tr>
<tr>
<td>hsp</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>HSR</td>
<td>Heat shock response</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HT</td>
<td>Hypoxanthine-thymidine</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JCA</td>
<td>Juvenile chronic arthritis</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NZB</td>
<td>New Zealand Black</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline-tween</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid Factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonuclear protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rotherwell Park Memorial Institute Essential Media</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviations</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethyl-ethylene diamine</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris-hydroxymethyl-propanediol</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Polyoxyethylyene-sorbitan monolaurate</td>
</tr>
<tr>
<td>TT</td>
<td>Thermotolerance</td>
</tr>
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</table>
WB  Washing buffer
μg  Microgram
μl  Microlitre
1.1 Heat Shock Proteins

General Introduction

The heat shock response was first identified in 1962 when Ritossa described the formation of chromosome puffs in the salivary glands of the fruitfly *Drosophila bucksii* subjected to temperature elevation, sodium salicylate or dinitrophenol [Ritossa 1962]. However it was not until 1973 that Tissières demonstrated that these 'puffing' patterns corresponded with the synthesis of a group of proteins, against a background of repression of general protein synthesis, which he named the heat shock proteins (hsp) [Tissières *et al.* 1974].

Since then it has been demonstrated that many types of stresses can induce increased synthesis of these proteins which are thus often referred to as stress proteins [Lindquist 1986]. It has been shown that some of these proteins are constitutively expressed whilst others are inducible by stress; and that they play critical roles in the cell in the unstressed state. In particular, they function in chaperoning proteins of the cell ensuring that they are maintained in their correct state under normal conditions [Lindquist 1986 Lindquist 1988].

Classification, structure and function

The hsp s have been classified into families based upon their molecular weight (MW). In mammals these are hsp100, hsp90, hsp70, hsp60, the 22-32kD hsp s and ubiquitin which has a MW of 7 to 8kD (table 1.1)

Hsp100
The hsp100 proteins comprise a highly conserved family that includes proteins from bacteria, yeasts and mammals. The family has been divided
Table 1.1. Classification of heat shock proteins into families

<table>
<thead>
<tr>
<th>Hsp</th>
<th>Members</th>
<th>functions/comments</th>
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<tbody>
<tr>
<td>Hsp104/110</td>
<td>Clp</td>
<td>required to survive severe stress</td>
</tr>
<tr>
<td>Hsp90</td>
<td>Gp96, hsp90α, hsp90β, Grp94, htpG</td>
<td>maintains proteins in inactive form until required, cellular kinases</td>
</tr>
<tr>
<td>Hsp70</td>
<td>hsp72, hsp73, hsc70, Grp78, dnaK (E.coli)</td>
<td>assembly of multimeric complexes</td>
</tr>
<tr>
<td>Hsp60</td>
<td>hsp63, hsp65, GroEl (E.coli)</td>
<td>molecular chaperone, major bacterial antigen</td>
</tr>
<tr>
<td>Hsp27</td>
<td>hsp25, hsp29, p24</td>
<td>estrogen responsive, drug resistance</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td></td>
<td>Protein degradation</td>
</tr>
<tr>
<td>Other Hsps</td>
<td>hsp32, 47, hsp56</td>
<td>heme-oxygenase, immunophilin</td>
</tr>
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</table>
into subfamilies, (ClpA, ClpB, ClpC) on the basis of two highly conserved nucleotide-binding domains [Gottesman et al. 1990]. The level of amino acid homology in these regions is remarkable, 50-80% identity with additional 20-40% homology. The proteins in the ClpB subfamily are all heat inducible and have higher homology with each other than with the constitutively expressed members of the family. This might imply that they share a conserved function in stress tolerance. A role for the heat-inducible hsp100 has been suggested by the activity of one of the constitutive members of the family, the *Escherichia coli* ClpA protein. ClpA functions as an ATP-dependent regulator of ClpP protease. Experiments measuring the breakdown of amino acid analog-containing proteins indicate that the Clp complex is responsible for about 15% of the turnover of abnormal proteins in *E. coli*, although the role of ClpP protease in the turnover of stress damaged proteins is unclear. There is also some evidence that a functional relationship exists between hsp104 and hsp70 [Sanchez et al. 1993]. Analysis of *S. cerevisiae* strains carrying multiple mutations in Hsp104 and hsp72 show that when hsp72 levels are reduced hsp104 becomes important for growth at normal and at moderately high temperatures. Conversely, when hsp104 levels are reduced, hsp72 becomes important in thermotolerance. These results suggest that hsp72 and hsp104 function in the same pathway or on parallel pathways that partially overlap.

HSP90

Members of the hsp90 family are present in prokaryotes, in the cytosolic/nuclear compartment of eukaryotes and also in the endoplasmic reticulum of higher eukaryotes. Deletion of the *E. coli* hsp90 gene (htpG)
has no effect on growth at normal temperatures and results in only a very subtle reduction in growth at high temperatures [Barwell and Craig 1988]. However, in *S. cerevisiae* hsp90 proteins are essential at all temperatures. Members of the hsp90 family interact with many other cellular proteins, including casein kinase II, the heme-regulated eLF-2α kinase, several steroid hormone receptors, oncogenic tyrosine kinases, calmodulin, actin, and tubulin [Morimoto *et al.* 1990 Sanchez 1990]. In addition hsp90 interacts with hsp56 and hsp70. Hsp90 is the most abundant constitutive mammalian hsp [Winfield and Jarjour 1991]. Hsp90 is a soluble cytoplasmic protein although it may also be found in the nucleus, especially upon heat shock [Lindquist and Craig 1988; Baulieu *et al.* 1990]. Hsp90 exists as a dimer *in vivo*, composed of two identical units. These dimers have been shown to exist in the cytosol complexed with other hsps, in particular hsp70 and hsp56 [Perdew and Whitelaw 1991]. There are two isoforms of hsp90, which are encoded by two different genes: α and β. There is constitutively about twice as much β as α protein [Moore *et al.* 1991]. The mRNA for hsp90α is 2.95kb and for hsp90β 2.7kb [Hickey *et al.* 1989; Simon *et al.* 1987]. There is a 97% homology between the two isoforms [Anderson 1989].

All eukaryotic hsp90s have a region with a high density of negatively charged amino acids located at the same relative position in the protein [Lindquist and Craig 1988]. There is also a smaller region of high negative charge density at the C-terminal end. The C-terminal end is the most divergent region, but the four most terminal amino acids (glu-glu-val-asp) are the same in all eukaryotic hsp90s, and are also found in eukaryotic hsp70s, which implies that this sequence serves an important purpose. Hsp90 appears to function primarily as an anchor in the cytoplasm for various target
proteins, with a role in rendering these proteins inactive until bound and activated by their respective ligands. The ability of hsp90 to bind actin and tubulin may provide a means of transporting proteins around the cell to their site of activation.

A number of virus transforming proteins are target proteins for hsp90. The Rous sarcoma virus transforming protein (pp60-src) upon synthesis is immediately associated with hsp90 and a 50kD protein complex, and at this stage pp60-src is phosphorylated at serine but not tyrosine residues. The 50kD protein has been shown to complex with hsp90 dimers in the cytosol [Whitelaw et al. 1991]. Pulse chase experiments show that pp60-src simultaneously loses its association with hsp90, is activated as a kinase, is phosphorylated at the tyrosine residues and is transported to the cell membrane. The half life of the complex (hsp90-pp50--pp60-src ) is 15 minutes. In transformation defective mutants of pp60-src this complex is stable, there is no association with the membrane. nor is there phosphorylation at tyrosines [Lindquist 1986].

Hsp90 has been shown to form stable complexes with other transforming proteins which have tyrosine kinase activity [Sorger and Pelham 1987]. It also forms complexes with other cellular kinases such as the heme-controlled eukaryotic initiation factor (eIF-2α kinase, which regulates translation in reticulocytes by phosphorylation of the smallest sub-unit of eukaryotic peptide initiation factor 2). Hsp90 co-purifies with this kinase and appears to modulate activity of the enzyme. Thus hsp90 may play a role in the regulation of protein synthesis since it effects increased phosphorylation of eIF-2α via the heme-sensitive kinase, and is therefore a potent inhibitor of protein synthesis in the reticulocyte system. It has been suggested that eIF-
2α phosphorylation is a mechanism which contributes to the inhibition of general translational activity which immediately follows heat shock [Rose et al. 1989]. Its ability to phosphorylate eIF-2α is removed if hsp90 is dephosphorylated, which might explain the finding that hsp90 possesses an ATP binding site and autophosphorylating activity together with a nucleotide binding site [Csermely and Kahn 1991; Gething and Sambrook 1992].

The involvement of hsp90 with steroid receptors was first identified when a 90kD protein was shown to be part of the 8S nontransformed, non DNA-binding glucocorticoid receptor (GR) and was found to be common to all steroid hormone receptors [Joab et al. 1984]. Hsp90 has been found to repress receptor function in a hormone dependent manner as assessed by DNA-binding [Dennis et al. 1988]. The high negative charge of hsp90 may be important in preventing the GR complex from binding to DNA, thus preventing the activation of steroid responsive genes [Sanchez et al. 1987]. The 8S GR binds to actin filaments via hsp90, which may serve as an anchoring mechanism [Miyata and Yahara 1991]. Association of hsp90 with the GR occurs late in translation or immediately following translation of GR mRNA [Dalman et al. 1989]. Hsp90 is required for the GR to bind steroid, and dissociation of hsp90 is sufficient to inactivate the unoccupied receptor [Bresnick et al. 1989; Housely et al 1990]. Thus hsp90 both inhibits DNA GR binding without steroid and improves ability to bind steroid when exposed to it. An exception to this may be the androgen receptor, where hsp90 is not apparently required for the high-affinity binding of steroid [Ohara-Nemoto et al. 1991].

A model has been postulated whereby hsp90 binds its hormone receptor via the ligand-binding domain thus masking the DNA-binding domain [Dalman
et al. 1991; Weigel et al. 1989]. The binding of the receptor to DNA occurs through a zinc finger motif [Freeman et al. 1988]. When hormone diffuses into the cytosol, it binds the receptor via the ligand-binding domain, producing a conformational change to the DNA binding domain, release of hsp90, and unmasking of the DNA binding site [Baulieu et al. 1990].

More recently, it has been shown that the 8S GR complex contains a 56kD protein and hsp70 as well as hsp90 [Sanchez 1990; Perdew et al. 1991]. Upon heat shock the complex is unchanged except that both constitutive and inducible forms of hsp70 are present. Evidence that hormone is necessary for the conformational change in the GR, enabling it to bind to DNA and therefore activate steroid-responsive genes, is demonstrated by the finding that dissociation alone of hsps90, 70 and 56 is not sufficient to generate a functionally active receptor [Bagghi et al. 1991]. Thus, when the anti-progestin drug RU486 is bound to the receptor, efficient transcription is prevented; although the precise mechanism of action of RU486 is known. Several models have been proposed whereby the drug stabilises the hsp90-progesterone complex, another suggests that RU486 effects the release of hsp90 but induces a conformational change which blocks the binding of the receptor to progesterone-responsive genes [Baulieu et al. 1990].

A 59kDa immunophilin (FKBP59) has also been shown to interact with hsp90 and hsp70 in the inactive GR complex [Tai et al. 1992]. Immunophilins are proteins that bind the immunosuppressants FK506, rapamycin and cyclosporin A and mediate the immunomodulatory effects of these drugs. As with hsps immunophilins are ubiquitous and are highly conserved throughout evolution. They may be involved in protein folding, assembly and transport. The authors proposed that FKBP59 may be involved in the
assembly of the inactive GR complex. It now seems that FKBP59 is the same as, or related to the hsp56 previously described.

Thus hsp90, complexed with different proteins as in the case of pp60-src and steroid receptors, has different roles in the stabilisation and transport of different target proteins [Whitelaw et al. 1991]. The universal theme appears to be the role of hsp90 in maintaining proteins in an inactive form until activation occurs by reaching the cell membrane in the case of pp60-src, or by binding of steroid ligand in the case of the GR. Hsp90 may be present in excess merely to ensure that all its target proteins are bound or it may well have another, as yet undefined, function. The finding that hsp90 is a murine tumour transplantation/rejection antigen (see section 1.2) is an indication that hsp90 may have various roles in the immune system [Moore et al. 1991; Ullrich et al. 1986]. In addition, hsp90 family members exhibit cellular and developmental stage-specificity and they may have a role in cell cycle regulation [Kohda et al. 1991; Gruppi et al. 1991].

Hsp70

Hsp70 genes are members of a multigene family whose members respond to elevated temperature or stress in different ways. The human hsp70 family has a 73% homology with its Drosophila homologue and 50% homology with the E. coli dnaK product [Lindquist 1986; Lindquist and Craig 1988]. There are four members of the human hsp70 family, all with fairly similar functions, the differences being in their inducibility and compartmentalisation [Welch 1991]. Hsp72 is the major heat-inducible hsp70, (also known as hsp70) it is also cell-cycle regulated and adenovirus protein inducible. Hsp73 (also known as hsc70, hsc73) is present at high levels constitutively in
differentiating cells. GRP78 (also known as immunoglobulin binding protein or BiP) is present in ER and lysosomes; there is also a 75kDa homologue (GRP75) present in mitochondria.

Mammalian hsp70s are methylated at arginine and lysine residues, bind ATP, are phosphorylated and exist as oligomers. Hsp72 and hsp73 are present in both the cytoplasm and nucleus, and after heat shock are associated with the granular region in nucleoli. Hsp70s are mainly involved in the disruption of molecular protein interactions such as in transport of proteins across membranes and binding of proteins in the endoplasmic reticulum (ER). Vesicles involved in cellular transport from one side of the membrane to another are coated with polymerised clathrin coats which are disassembled by hsp73 [Brodsky et al. 1991]. Hsp73 also plays an important role in lysozomal proteolysis which is selective for proteins containing the sequence lys-glu-arg-gln. Hsp73 binds to this region and facilitates transfer into the lysosome [Dice et al. 1990]. Hsp73 has also been shown to bind to mutant forms of p53 [Pinhasi-Kimhi et al. 1986]. All these reactions require the hydrolysis of ATP and the high affinity of hsp70s for ATP allows this energy to be generated [Flaherty et al. 1990]. A model for the functions of hsp70 suggests that they bind to the hydrophobic region of proteins which occur naturally or are presented as a result of protein denaturation and stabilise them in a fully or partially unfolded state [Pelham 1988]. Binding to hsp70 prevents or disrupts inappropriate protein-protein interactions which might result in misfolding or aggregation, and is reversed by ATP hydrolysis. The term 'molecular chaperones' initially used to describe hsp65 has also been used to describe the hsp70 family [Ellis 1987]. This term originally defined proteins involved in ensuring or facilitating the correct folding and assembly
of oligomeric proteins complexes.

HSP60

The hsp60 proteins are present in the cytosol of bacteria (where they are termed GroEl proteins), in the matrix of the mitochondria and in the stroma of chloroplasts (where they are known as chaperonin-60 proteins). They are among the most abundant cellular proteins in bacteria at normal temperatures. Most information about the structure and function of hsp60 comes from studies of the *E. coli* homologue. From this model it seems that hsp60 is composed of a common oligomeric structure of two seven-membered rings stacked on top of each other. This structure provides multiple binding sites for a single polypeptide chain [Gething and Sambrook 1992]. Hsp60 binds ATP with high affinity and has weak ATPase activity, this ATPase activity is modulated by a co-chaperonin [Goloubinoff *et al.* 1989]. Hsp60 functions as a molecular chaperone, promoting the folding of newly synthesised bacterial proteins as well as eukaryotic proteins translocated into the mitochondria and chloroplasts [Horowich *et al.* 1993].

Hsp56 is a peptidyl prolyl isomerase and an immunophilin [Chambraud *et al.* 1993; Tai *et al.* 1982]. Although there is no known sequence homology with hsp60 at the N-terminal end, it does have certain features in common with hsp60. It is present in the cell at low levels, is expressed constitutively and forms large heteromeric complexes. Thus hsp56 may perform a similar function to hsp60, being involved in the the assembly of protein -8S GR complexes. A major interest in hsp60 is the possible involvement in autoimmunity in humans of the mycobacterial homologue, this will be discussed in section 1.2.
HSP27

The small hsps of which hsp27 is the human equivalent are a group of heterogeneous proteins and are the least conserved hsps between species. Hsp27 purified from HeLa cells is a large homo-oligomer and electron microscopy shows a ring-like structure [Arrigo and Welch 1987]. Hsp27 is localised in the peri-nuclear cytoplasm and is closely related to the Golgi complex. Heat shock results in hsp27 accumulating into large complexes. This is similar to the aggregation formed by the lens α crystallin proteins, and there is significant homology between hsp27 and these proteins. Hsp27 was identified initially in human breast tumour cells as an estrogen responsive protein [Edwards et al. 1981, Fuqua et al. 1989, Ciocca and Luque 1991]. Early studies showed that in MCF-7 cells hsp27 is expressed constitutively and that its synthesis increases depending on both duration of estrogen exposure and dose and requires the interaction of the hormone with the estrogen receptor [Edwards et al. 1981]. Hsp27 also appears to have important functions in drug resistance in breast cancer cell lines [Oesterreich et al. 1990]. The relationship of hsp27 and cancers will be discussed in section 1.2.

Ubiquitin

Ubiquitin, a 7-8 kDa protein comprising of 76 amino acids, was first isolated from calf thymus [Goldstein 1975]. It exists either free or covalently linked to a wide variety of cytoplasmic nuclear and membrane proteins [Finley et al. 1985]. It is a compact globular protein [DiStefano and Wand 1987] and is highly conserved between all species. It is a constitutive protein but its levels
can rise five to seven fold after stress has been induced. One of the main roles of ubiquitin in the cell appears to be in the process of ATP-dependent protein degradation. Here the relevant proteins must be attached to ubiquitin before they can undergo proteolysis [Ciechanover 1994]. This pathway involves at least three different ubiquitin enzymes: E1, E2 and E3, some of which are induced by heat shock and other stresses. In the first step of the pathway, ubiquitin is activated in an ATP-dependent reaction by a ubiquitin - activating enzyme (E1). The activated ubiquitin molecule is transferred to a ubiquitin conjugating enzyme (E2). Finally the activated ubiquitin molecule is transferred to a protein substrate, sometimes with the help of a ubiquitin protein ligase (E3). An isopeptide linkage is formed between the carboxyl terminus of the ubiquitin molecule and an internal lysine residue of the substrate protein. Targeting for protein degradation requires the presence of a long chain of ubiquitin molecules attached to a substrate linked by ubiquitin conjugating enzyme. This chain of ubiquitin molecules allows the substrate protein to be recognised and degraded by the 26S protease, a large multi-subunit ATP-dependent proteolytic complex in the cytosol. The ubiquitin molecules are then released and recycled for reactivation and reuse. In the nucleus ubiquitin attaches to histone H2A and H2B. This is a reversible modification that occurs preferentially in active transcribed regions of chromatin after core assembly.
The Heat Shock Response (HSR)

When cultured cells or whole organisms are exposed to elevated temperatures or other stresses, they respond by synthesising a group of highly conserved proteins. This response is universal and has been found in every organism studied. The induction temperature for the HSR varies between species. The maximal response occurs 10-15°C above the optimum growth temperatures for organism which grow over a broad range of temperatures and about 5°C above the optimum growth temperature for organisms which grow over a narrow range. The HSR is also influenced by factors such as the metabolic state of the cell, previous incubation temperature and the rate of temperature change [Lindquist 1986].

Thermotolerance

Study of the HSR has led to the idea of 'thermotolerance' (TT), whereby cells exposed to moderately elevated temperatures are more likely to survive when exposed to further, higher temperatures than those that not been pre-exposed. This tolerance also extends to other forms of stress and cross-tolerance has also been reported where tolerance to different stresses occurs following exposure to one stress [Jenkins et al. 1988]. The mechanism of TT is complex, and not simply an increase in cell survival as a result of increased hsp expression. Inhibition of hsp induction in mammalian cells by the addition of cyclohexamide treatment during heat shock (HS) does not block the development of TT [Burdon 1987]. Alternatively there could be some other cellular component involved as well. There is some evidence to suggest that hsps are constitutively involved in the resistance to heat shock. A mouse cell line over expressing anti-sense hsp90 mRNA has
lower survival upon HS, but is no more sensitive to herpes simplex virus infection than parental cells [Bansal et al. 1991]. Overexpression of human hsp70 using expression constructs is thermoprotective in CV1 monkey cells and rodent cells [Angelidis et al. 1991]. This was also reported to occur in cells expressing mutant hsp70 in which the ATP-binding domain has been deleted [Li et al 1992].

The cell cycle

Cellular growth, proliferation and differentiation involve the synthesis and transport of large quantities of new proteins within the cell. Thus it is likely that hsp70 have important functions in growth and differentiation.

Expression of hsp90 shows cellular and developmental stage specificity in mouse germ cell differentiation and embryogenesis [Gruppi et al. 1991]. Differentiation of mouse embryonal carcinoma F9 cells is associated with an increase in hsp86 mRNA, which is maintained if differentiation is maintained [Kohda et al. 1991]. The effect of ageing upon the cell cycle appears to be mediated via hsp70. There is a decreased proliferative response to the mitogen phytohaemagglutinin (PHA) in lymphocytes from aged donors, and this is paralleled by decreased induction in the pre-replicative phase of hsp90 and hsp73 [Fassen et al. 1989]. These hsp70 are normally highly induced in mitogen-activated T cells. These data could imply that in vivo ageing of human T cells may cause a defect in the induction of gene products required for transition from quiescence to the replicative phase of the cell cycle. Chemicals have been shown to induce developmental defects in mammals and the same chemicals can also induce hsp70. These defects have been shown to be preventable by the induction of thermotolerance at
certain critical stages of development [Peterson 1990].

Control of hsp expression

In prokaryotes such as *E. coli*, the major hsps are coded by single genes expressed constitutively at all temperatures [Lindquist and Craig 1988]. Following a temperature shift or treatment with cell damaging agents, such as ethanol, the rate of expression of these genes accelerates. After a few minutes the rate of expression subsides reaching a new steady state level, characteristic of the new temperature. The HSR is regulated positively at the transcriptional level by the σ^{32} polypeptide which is the product of the rpoH (hptR) gene. Subsequently a further set of HS genes was discovered also positively regulated at the transcriptional level by the σ^{24} polypeptide. There appears to be a network of expression control between the two pathways since one of the promoters of the rpoH (hptR) gene is transcribed by the σ^{24} RNA polymerase enzyme. In eukaryotes, the HS response is controlled mostly at the transcriptional level by a positively acting heat shock factor (HSF). HSFs bind HSE, a specific DNA recognition sequence located upstream of HS gene promoters.

Heat Shock Factors

In the unstressed cell HSFs are present in both the cytoplasm and the nucleus. In response to HS or other physiological stresses HSF assembles in a trimer and accumulates within the nucleus. This response is rapid: activation and binding of HSF to the HSE can be detected within minutes of temperature elevation [Sarge 1993, Westwood 1991]. Although the kinetics and magnitude of DNA binding activity are often proportional to the
transcriptional response, HSF DNA binding activity does not always correlate with transcriptional activity, suggesting that there are multiple steps in the activation process [Abravaya 1991, Jurvich 1992]. Moreover HSF exhibits a stress-dependent phosphorylation that may modulate its transcriptional activity [Sarge 1993, Larson 1988]. This heat shock transcriptional response lessens upon prolonged exposure of cells at intermediate temperature or upon return to physiological temperature. This lessening is accompanied by the conversion of the active trimeric form of HSF to the non-DNA binding monomer and by a return to the normal distribution. In contrast, prolonged exposure to extreme temperature results in sustained HS gene transcription and HSF DNA binding activity.

This stress dependent conversion of HSF to its active DNA binding form suggests that HSFs are negatively regulated. This regulation is not an intrinsic property of the protein as expression of recombinant Drosophila, chicken mouse and human HSFs in E. coli yields a constitutively active DNA binding factor [Nakai 1993, Rabindran 1991]. It is possible that the DNA binding ability of HSF in eukaryotes is controlled by a regulatory protein not present in E. coli. It has been speculated on the basis of early studies with Drosophila and yeast, that heat shock proteins themselves may negatively regulate heat shock gene expression via an autoregulatory loop. Thus, the increased levels of misfolded proteins induced during stress could result in the activation of HSF. There is evidence to suggest that this can occur since the heat shock transcriptional response correlates with increased levels of denatured proteins [Morimoto 1992]. Similarly, activation of heat shock transcription in human cells by intermediate elevated temperatures is blocked by incubation with protein synthesis inhibitors, suggesting that
misfolding of nascent polypeptides may somehow trigger the response.

Molecular chaperones such as hsp70 may be important in autoregulation of the heat shock response. They have been shown to facilitate protein folding by stabilising intermediate folded states of nascent proteins, thus preventing them from engaging in inappropriate interactions that may lead to irreversible, nonspecific aggregation. Several in vitro experiments support this idea of a regulatory role for hsp70 in HSF activation. Inactive HSF in cytoplasmic extracts from non-heat shocked HeLa cells can be converted into the DNA binding state by exposure to heat, alteration in pH or by non-ionic detergents [Mosser 1990], but the addition of hsp70 stops this conversion [Abravaya et al. 1992]. The effect of hsp70 can be reversed by the addition of adenosine triphosphate (ATP), an essential feature of heat shock protein function. These results imply that inhibition might be mediated through hsp70 possibly through alteration of the native conformation of HSF. However a stable interaction between hsp70 and the inactive form of HSF has not yet been directly demonstrated. Complexes containing hsp70 and the active trimeric form of HSF have been detected in extracts of heat shocked cells [Abravaya 1992]. This association may be important in the conversion of the HSF active trimer to monomers, a key event in the lessening of the heat shock transcriptional response.

It is also quite possible that other hsp7s are involved HSF regulation. A suitable model for regulation is as follows. When the cell is not stressed HSF is maintained in a non-DNA binding form through transient interactions with hsp70 that possibly act by stabilising a specific conformation of HSF. During HS, the presence of denatured proteins creates a large group of new protein substrates that compete with HSF for association with hsp70. Therefore HS
and other stresses initiate events that remove the negative regulatory influence on HSF DNA binding activity. The free HSF can then assemble into trimers and bind to DNA, leading to elevated transcription, synthesis and accumulation of heat shock proteins, in particular hsp70. Association of HSF with hsp70 may be important for regulation of its transcriptional activity or conversion back to the monomeric form.

More recently it has been shown that there is a family of HSFs containing at least three members, all of which share some structural features for example a DNA-binding domain at the NH2-terminus, and adjacent clusters of hydrophobic amino acids organised into leucine zippers [Sarge et al. 1991, Schuetz et al. 1991, Rabindran 1991 et al.,]. All three HSFs are expressed simultaneously in most cells, and the DNA binding activity of each is negatively regulated [Nakai and Morimoto1994].

Rapid synthesis of hsp90 is also effected at the post-transcriptional level. The mRNAs of hsp genes in general lack introns, thus by-passing the need for these to be spliced out prior to translation. However hsp90 does possess introns in both the hsp90α and hsp90β forms [Rebbe et al. 1989]. Studies of hsp83 in *Drosophila* demonstrated that it is synthesised to high levels constitutively and at moderately elevated temperatures but not at high temperatures. The block at high temperatures occurs due to a failure in RNA processing, with failure to splice out the introns [Yost et al. 1990]. The high constitutive level of hsp90 expression might suffice to deal with the effects of severe heat shock. Three of the six HSEs in the mammalian hsp90β are located in the first intron. It might be evolutionarily disadvantageous for this gene to lose its introns together with its potential for rapid transcription through activation of HSEs [Rebbe et al. 1989].
Degradation of mRNA is another means of regulating hsp synthesis. This seems to be particularly applicable to the hsp72 gene. The rate of synthesis of hsp72 is coupled to the degradation of its mRNA under normal conditions, and the 3' untranslated region of hsp72 mRNA plays a critical role in its own degradation. Thus, at normal temperatures, hsp72 mRNA is unstable, but upon heat shock is stabilised [Yost et al. 1990].
1.2 Heat Shock Proteins: Infection and Autoimmunity

The Immune System

A major factor in the development of the immune system is the continuous encounter with a seemingly infinite diversity of invading pathogens. The immune system deals with this diversity by generating an equally large number of receptors capable of discriminating between these structures. Antibodies produced by B lymphocytes recognise their counterparts (antigens) directly, whereas T cell receptors on the surface of T lymphocytes facilitate their indirect recognition of antigens.

Antigen recognition by T cells requires intracellular processing of microbial components by antigen presenting cells and subsequent presentation on the cell surface by specialised products that are coded by the major histocompatibility complex (MHC). T cells are therefore highly suitable for challenging microbes that live inside host cells. Microbial invaders and mammalian host cells are composed of many components which are highly conserved at the molecular level. Thus the immune system must differentiate between self and non-self structures. This is achieved by deletion or inactivation of those lymphocytes which express receptors for self structures at an early stage of development [Marrack and Kappler 1993]. Often, B-lymphocyte responses require help from T-lymphocytes; therefore prevention of recognition is restricted to T cells. The functional inactivation of T cells is initiated in the thymus and is maintained in the periphery through different mechanisms [Schwartz 1989]. These mechanisms are usually successful but recognition of self antigens can occur [Steinman 1993]. Whereas a limited 'amount' of autoimmunity is 'permitted' viz naturally occurring
autoantibodies, under normal conditions this is tightly controlled. When this control is lost in the appropriate genetic, hormonal or environmental setting a clinically overt autoimmune disease can develop. This could happen when either extrathymic development of some T cells occurs, or some self antigens fail to be presented in the thymus.

Heat shock proteins (hspS) have several features that might pose difficulties to the immune system. Hsps are amongst the most abundant intracellular proteins and are further elevated under stress [Lindquist 1986; Lindquist and Craig 1988]. Microbes entering the cell can result in increased synthesis of the hsps [Kaufmann 1990]. In addition, hsps are highly conserved across species. Therefore as a result of their abundance and high homology, hsps appear to be appropriate targets for the immune response. This targeting on a small number of similar proteins enables the immune system to deal with the whole spectrum of infectious agents. However, inappropriate cross reactivity with host cell hsps may result in autoimmune responses.

Types of Infectious agents

Microorganisms have utilised a variety of different strategies for invasion and infection of the host. Some live in extracellular spaces where they can secrete toxic components that are harmful to the host. Antibodies are responsible for both attacking the infectious agents and neutralisation of their toxic products [for a review see Paul and Seder 1993]. Usually, such microbes result in acute infections and are readily eradicated from the host once specific immunity has reached its maximum. There are also intracellular pathogens which evade antibody recognition. However, during their intracellular life, microbial proteins are degraded and presented on the
cell surface by MHC molecules, which can be recognised by T cells. In response to these changes, microbial pathogens produce increased hsp levels that could become early targets for the immune response. This has been shown by anti-hsp70 antibody or T cell responses that have been observed in infections with intracellular and extracellular infections [Haregwoin et al. 1989, Koga et al. 1989, Shanafelt et al. 1991]. Viruses must utilise host cell machinery in order to replicate. Free virus particles are potential targets for the immune system, but during intracellular replication and assembly viruses are inaccessible to antibodies. However, virus-infected host cells may be detected by T cells because viral proteins undergo processing and presentation by MHC molecules [Kaufmann and Reddehase 1989]. Although viral genomes do not appear to contain genes that code for hsp; viral infection or "infection " with prions has been shown to elevate host hsp synthesis [Doherty et al. 1992; Diedrich et al. 1993]. Therefore it is possible that viral infections could result in immune responses against the host's hsp.

Heat Shock Proteins and Infectious diseases.

Immune responses to hsp have been described in many infectious diseases. These are summarised in Table 1.2.1 [Kaufmann 1990; Young 1989; Young and Elliot 1991]. One of the first studies to investigate immune responses to hsp in infection identified hsp60-specific CD4+ T cell clones derived from patients with leprosy and from BCG-vaccinated healthy individuals [Emmrich et al. 1986]. High frequencies of CD4+ T cells reactive with hsp60 have been isolated from mice immunised with mycobacteria [Kaufmann et al. 1987]. In addition, vaccination of young children with the trivalent vaccine against tetanus, diphtheria, and pertussis induced high titres
Table 1.2.1 Heat shock proteins as targets of infectious agents. (Adapted from Kaufmann 1990)

<table>
<thead>
<tr>
<th>HSP</th>
<th>Infectious Agent</th>
<th>Disease</th>
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<tbody>
<tr>
<td>hsp90</td>
<td><em>Plasmodium falciparium</em></td>
<td>Malaria</td>
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<td></td>
<td><em>Trypanosoma cruzi</em></td>
<td>Chagas' disease</td>
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<td></td>
<td><em>Trypanosoma brucei brucei</em></td>
<td>Trypansomiasis of cattle</td>
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<td></td>
<td><em>Leishmania amazonensis</em></td>
<td>Leishmania</td>
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<td></td>
<td><em>Schistosoma mansoni</em></td>
<td>Schistosomiasis</td>
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<tr>
<td></td>
<td><em>Candida albicans</em></td>
<td>Candidasis</td>
</tr>
<tr>
<td>hsp70</td>
<td><em>Plasmodium falciparium</em></td>
<td>Malaria</td>
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<td></td>
<td><em>Trypanosoma cruzi</em></td>
<td>Chagas' disease</td>
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<td>Trypansomiasis of cattle</td>
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<td></td>
<td><em>Leishmania amazonensis</em></td>
<td>Leishmania</td>
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<td></td>
<td><em>Schistosoma mansoni</em></td>
<td>Schistosomiasis</td>
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<td></td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Tuberculosis</td>
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<td></td>
<td><em>Mycobacterium leprae</em></td>
<td>Leprosy</td>
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<td></td>
<td><em>Borrelia burgdorferi</em></td>
<td>Lyme disease</td>
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<tr>
<td>hsp60</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Tuberculosis</td>
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<td><em>Mycobacterium leprae</em></td>
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<td></td>
<td><em>Borrelia burgdorferi</em></td>
<td>Lyme disease</td>
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<td></td>
<td><em>Treponema pallidum</em></td>
<td>Syphilis</td>
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<td></td>
<td><em>Legionella pneumophila</em></td>
<td>Legionnaire's disease</td>
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<td>Q-fever</td>
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<tr>
<td>Small hsp</td>
<td><em>Schistosoma mansoni</em></td>
<td>Schistosomiasis</td>
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<td></td>
<td><em>Mycobacterium tuberculosis</em></td>
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<td></td>
<td><em>Mycobacterium leprae</em></td>
<td>Leprosy</td>
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of anti-hsp antibodies [Del Giudice et al. 1993]. These findings, together with those listed in table 1.21 are thought to imply that hsp are immunodominant antigens in infections with a variety of pathogens in several species. The identification of hsp-reactive T cells and antibodies in healthy individuals imply that immune responses are already induced by contact with low virulence pathogens capable of invading and surviving in the host for a restricted time without causing clinical disease [Kaufmann et al. 1990]. Frequent encounters with these pathogens focus the immune response to hsp regions conserved within these micro-organisms. As a result of these encounters with conserved epitopes, immune responses to hsps may become elevated above those to species-specific antigens which are contacted less frequently.

Micro-organisms that invade the host are exposed to multiple adverse stimuli such as alterations in pH, temperature and pO$_2$ as well as mechanisms such as phagocytosis [Kaufmann et al. 1990; Kaufmann et al. 1991]. In order to survive these adverse surroundings, microbial pathogens activate various mechanisms, including increased expression of hsps. The importance of hsps for bacterial survival has been demonstrated by deletion of the hsp gene in *Salmonella typhimurium*. These mutants succumb to macrophage killing and lose their virulence in mice [Fields et al. 1986; Johnson et al. 1991].

Interaction between host and pathogen induces elevated synthesis of hsps in both, including in human host cells [Kaufmann et al. 1991]. Although increased hsp synthesis contributes to protection of host cells, abundant synthesis of self-hsp might transform these cells into targets for the anti-hsp
immune responses [Kantengwa et al. 1991; Koga et al. 1989; Kaufmann et al. 1991]. In this way immune responses following infection may be pathogenic rather than protective, and some evidence of this does exist [Mistry et al. 1992; Ford et al. 1993]. It is also possible that elevated hsp production could alter the balance between an efficient response to infection and an inappropriate response leading to autoimmunity.

Heat Shock Proteins and Autoimmunity

Hsp expression (caused by various cellular stresses including inflammation, infection and trauma) could serve as a universal indicator of stressed cells. Hsps have also been shown to be induced by cell activation and are involved in protein synthesis in activated cells [Kaufmann et al. 1991]. Recognition of activated immune cells could be an integral part of a regulatory immune network whereby T cells promote down regulation of ongoing immune responses and thus prevent immune hypereactivity [Cohen 1992]. Increased hsp synthesis in stressed or activated cells is followed by hsp degradation, which may lead potentially to higher levels of cytoplasmic hsp peptides being available for presentation by the MHC pathway. It has also been suggested that the immune system may be biased towards hsp and other dominant self antigens [Cohen and Young 1991]. These authors postulate that autoimmunity to self hsp is the natural event that increases the speed and strength of the anti-infectious immune response by virtue of the similarity between the invading and host hsp. Thus, autoimmunity may be the result of improperly regulated immunity to self-hsp or other dominant autoantigens [Cohen and Young 1991].

Recognition by the immune system of stressed cells could be achieved by
two kinds of hsp expression.
1. Presentation of hsp peptides by MHC molecules on the cell surface. This allows recognition by CD4 or CD8 α/β T cells and at least some γδ T cells.
2. Surface expression of the hsp. This allows recognition of stressed cells by antibodies and, possibly γδ T cells.

MHC-restricted presentation of self heat shock protein

The first evidence that CD4+ T cells recognise self-hsp in the context of MHC class II gene products came from studies with synthetic peptides. In these experiments, peptides representing regions of autologous hsp60 were added to cultures containing antigen presenting cells and hsp-specific T cells [Lamb et al. 1989; Munk et al. 1989]. Peptide recognition by T cells demonstrated that T cells with specificity for self-hsp are not deleted during thymic development, but it was not proven that the physiological presentation of hsp epitopes was derived from endogenous hsp. Evidence that this may occur came from studies showing that hsp60-reactive T cell lines and clones selectively recognise stressed host cells [Koga et al. 1989]. These studies demonstrated that T cells directed against mycobacterial hsp60 were CD8+ and MHC class I restricted. Increased levels of hsp60 were detected in these cells [Kaufmann et al. 1991]. Importantly, treatment with antisense oligonucleotides against specifically abolished recognition of stressed cells by the hsp60 reactive CD8+ T cells [Kaufmann and Schoel 1994]. These findings suggest that host cells might present peptides derived from self-hsp under stressed conditions.

Several groups have established γδ T cell hybridomas from the thymuses of newborn mice and the spleens of naive adult mice, and found that some of
these γ/δ T cells recognise the mycobacterial hsp60 and in particular, are specific for amino acids 180-188 [O'Brien et al. 1992; Born et al. 1990; Fu et al. 1993]. Although this region shows only partial sequence homology with its mammalian cognate protein, a peptide representing the homologous sequence of the mammalian hsp60 was able to stimulate these γ/δ T cells. These results suggest that a high frequency of γ/δ T cells may recognise self hsp60 peptides.

Heat Shock protein peptides eluted from MHC Gene Products

MHC class I and II gene products are responsible for the presentation of antigenic peptides to T lymphocytes. Both MHC molecules form a pocket in which peptides are accommodated and presented to T lymphocytes. MHC class I presents peptides recognised by CD8+ T cells and MHC class II presents peptides recognised by CD4+ cells. More recently it has become possible to elute the naturally processed peptides from MHC molecules and determine their sequence [Jardetzky et al. 1991; Rammensee et al. 1993]. These studies have clearly demonstrated that hsp-derived peptides are presented by cells within the context of MHC class I and class II molecules. Since hsps are cytoplasmic proteins, presentation in the context of MHC class I gene products would not be unexpected. However, hsp peptides were also eluted from MHC class II molecules, clearly demonstrating that cytosolic proteins are loaded into both pathways, and that hsp peptides are not excluded from antigen presentation to T cells.

Thus, the identification of T cell clones and lines capable of recognising synthetic peptides consisting of sequences of self-hsp suggest that such epitopes can be the target of T cell responses. Studies have been
undertaken to look at sequence homologies between peptides eluted from MHC molecules and proteins from infectious agents. One such analysis demonstrated that an hsp70 peptide from a human MHC class II molecule was almost identical to hsp70 molecules from various pathogens; and the hsp peptides eluted from the MHC molecules also showed more than 55% similarity to unrelated proteins [Chicz et al. 1993].

The question then arises of how tolerance against such naturally presented self-epitopes including those of hsps is achieved. Since self-hsp specific T cells have been detected it seems that not all self-reactive T cells are deleted in the thymus. Those self reactive T cells that evade thymic deletion must be silenced via peripheral mechanisms. Possibly, tolerance to the naturally presented hsp epitopes could result from oral tolerisation by cross reactive hsp peptides from food [Mowat 1987; Schwartz et al. 1989]. Therefore, highly conserved hsp regions might be presented to the T cell system through three different routes: natural processing of self-hsp; food uptake; and infection. It is possible that these different routes could markedly influence the immune response; food derived hsps could contribute to tolerance; whereas infection promotes active immunisation. The development of autoimmune disease may therefore depend on a balance between the different types of antigen deliveries. Autoimmune disease may be facilitated by frequent encounters through infection.
Heat Shock Proteins And Autoimmune disease

At present the role of hsps in the development of autoimmune disease remains unclear; the role of hsps in SLE will be discussed in section 1.4 [Leung and Gershwin 1991]. One hypothesis was that self-hsp-reactive T cells and antibodies are primarily stimulated by cross reactive epitopes derived from infectious agents usually through chronic infection. The abundant presence of cross reactive epitopes was considered sufficient to break tolerance against self. Thus the antimicrobial immune response would be directed against host cells overexpressing hsp as a result of stress. Alternatively, T cells with specificity for conserved epitopes may be attracted to a particular tissue site and then recognise cross-reactive epitopes derived from unrelated, tissue specific antigens resulting in an organ-specific autoimmune disease [Jones et al. 1993, Kaufmann and Schoel 1994]. The concept that hsps of microbial origin can act as the inducing antigen may need to be re-evaluated. It is possible that hsp overexpression at the site of inflammation occurs as a result of tissue destruction and responses caused by T cells recognising tissue-specific antigens. Under these circumstances hsp reactive T cells would arrive secondarily and further exacerbate local tissue destruction but would not be responsible for inducing autoimmune disease. Some evidence consistent with this theory has been described in experimental models [Doherty et al. 1992; Haas et al. 1992; Kaufmann et al. 1993]. The presence of hsp-reactive T cells would then be an ineffectual attempt of the immune response to restrain exacerbated immune responses.

The frequent identification of hsp-reactive T cells and antibodies in various autoimmune diseases has led to widespread speculation about the role of hsps as antigens in triggering or perpetuating these diseases. This idea was
further supported by the observation of increased hsp expression in cells present in inflammatory lesions (summarised in table 1.2.2). Table 1.2.3 summarises the evidence that hsps may play a role in animal models of autoimmune disease.

Substantial evidence has been provided for the participation of hsps in two experimental animal models: adjuvant arthritis (AA) of rats and insulin dependent diabetes mellitus (IDDM) of nonobese diabetic (NOD) mice [Van Eden et al. 1988; Boog et al. 1992; Cohen 1991; Elias et al. 1990, 1991]. Administration of complete Freunds adjuvant containing mycobacteria causes an autoimmune disease that closely resembles rheumatoid arthritis. T cells isolated from such mice recognise a non-conserved region of the mycobacterial hsp60. These T cells are capable of transferring either induction or protection against adjuvant arthritis [Yang et al. 1990, van Eden et al. 1988]. In addition, pre-immunisation of rats with soluble hsp60 or an hsp60 peptide renders animals resistant to subsequent attempts to induce adjuvant arthritis. It was claimed originally that hsp60 specific T cells have major roles in the development of reactive arthritis [Herman et al. 1991; Life et al. 1991]. Although T cells with specificity for non-conserved mycobacterial epitopes as well as conserved epitopes have been described, more recent findings indicate that the proportion of these hsp-specific T cells in lesions is small [Life et al. 1991].

In NOD mice a diabetic disease develops spontaneously which closely resembles IDDM in humans [Cohen and Young 1991; Elias et al. 1990, 1991]. Hsp60 specific T cells were found to be capable of transferring disease to recipients. However in this case the T cells recognised a conserved region of hsp60
Table 1.2.2 Studies with increased hsp expression in inflammatory diseases

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial Lining</td>
<td>^aRheumatoid juvenile Arthritis (Juvenile Chronic Arthritis)</td>
</tr>
<tr>
<td>Cartilage-pannus junction</td>
<td>^bRheumatoid arthritis</td>
</tr>
<tr>
<td>Oligodendrocytes</td>
<td>^cMultiple Sclerosis</td>
</tr>
<tr>
<td>B Cells</td>
<td>^dSystemic Lupus Erythematosis</td>
</tr>
<tr>
<td>β-cell secretory granules</td>
<td>^eInsulin Dependent Diabetes Mellitus</td>
</tr>
<tr>
<td>Thyroid Cells</td>
<td>^fGraves Disease</td>
</tr>
</tbody>
</table>

b: De Graeff-Meeder et al. (1990)
c: Freeman et al. (1992), Selmaj et al. (1991)
d: Norton et al. (1989), Erkeller-Yuksel et al. (1992)
e: Jones et al. (1990), Brudzynski et al. (1992)
f: Heufelder et al. (1991,1992)
Table 1.2.3. Major Evidence for heat shock protein Involvement in autoimmune diseases: Animal Models (adapted from Kaufmann and Schoel 1994).

<table>
<thead>
<tr>
<th>Disease</th>
<th>Evidence ( \text{(pro and contra hsp involvement)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental IDDM of NOD (^a) mice</td>
<td>( \text{Pro: hsp60-reactive T cells confers disease, prevention} ) with peptide</td>
</tr>
<tr>
<td>Adjuvant arthritis Lewis rats</td>
<td>( \text{Pro: hsp60-reactive T cells confer disease; epitope of unique for mycobacterial hsp60 amino acids 180-188} ) ( \text{Pro: prevention by immunisation with epitope amino acids 180-188} ) ( \text{Pro: prevention by immunisation with recombinant vaccinia virus expressing mycobacterial hsp60} )</td>
</tr>
<tr>
<td>Pristane induced arthritis (mice)</td>
<td>( \text{Pro: prevention by immunisation with mycobacterial hsp60} )</td>
</tr>
<tr>
<td>Adjuvant-induced arthritis (mice)</td>
<td>( \text{Pro: T cells reactive to mammalian hsp60 amino acids 412-423 acute inflammation} )</td>
</tr>
</tbody>
</table>

\(^a\) insulin dependent diabetes mellitus, \(^b\) non-obese diabetic
shared by both mammalian and mycobacterial hsp60. Increased expression of hsp60 has been observed in pancreatic β cells providing further evidence for hsp involvement in IDDM [Brudzynski et al. 1992]. Moreover the spontaneous development of IDDM in NOD mice can be prevented by immunisation with soluble hsp60 or the relevant peptide [Cohen 1991, Elias et al. 1992]. However, it now seems that the inducing antigen is the protein glutamic acid decarboxylase [Kaufmann et al. 1993, Tisch et al. 1993].

Although the mechanisms of involvement of hsps in these experimental autoimmune diseases differs, as indicated by the involvement of different hsp epitopes (unique and conserved in AA and IDDM respectively), they provide strong evidence of a critical role for hsps in the development of experimental autoimmune diseases. However, recent work has suggested that organ-specific antigens play an important role in these diseases with hsps having a much more minor role.

Initially hsp60 was proposed to have a central role in human IDDM, but more recent evidence suggests a minor role (if any) in this disease [Baekeshov et al. 1990]. It seems probable that as in the mouse, the autoantigen is glutamic acid decarboxylase which beyond having a similar molecular weight is totally unrelated to the hsp family. However, recent identification of amino acid sequence similarities between hsp60 and glutamic acid decarboxylase could be taken as circumstantial evidence for a relationship between these two antigens in IDDM [Jones et al. 1993].

Surface staining of macrophages derived from the joint fluid of patients with rheumatoid arthritis (RA) by mAbs to human hsp70 and hsp90 has been
demonstrated [Kaufmann et al. 1990]. IgG antibody titres to mycobacterial hsp60 are significantly elevated in RA compared to healthy individuals [Tsoulfa et al. 1989]. However, there is significant evidence that mycobacteria do not play an important role in RA. It is difficult to isolate T cell clones from synovial fluid or tissue from patients with RA which respond to mycobacterial hsp60. The frequency of hsp60-reactive T cell clones is at least as common in reactive arthritis, suggesting that it is not a disease-specific response [Life et al. 1991]. Thus the concept that hsps might be important in the pathogenesis of adult RA has become less tenable [De Graeff-Meeder et al. 1991; Res et al. 1991]. A summary of evidence of hsp involvement in human autoimmune diseases is described in table 1.2.4.

Surface expression of heat shock proteins

As intracellular proteins, hsps are likely to be sequestered from humoral and cellular immune interactions. However, recently several studies report that surface expression of hsps can be detected. The results from these experiments are summarised in table 1.2.5. These experiments included analyses of labelled cells with microfluorimetry, in situ staining of tissue samples and immunoprecipitation of surface molecules. Cytofluorimetric analyses and immunoprecipitation provided strong evidence for hsp60 expression by stressed macrophages [Wand-Wüttenberger et al. 1991]. Evidence has also accumulated for hsp60 expression by the Daudi B cell line and increased surface expression of an hsp70 by murine splenic B cells [Fisch et al. 1990; Pierce et al. 1991; Kaur et al. 1993].

Surface expression of several hsps have been reported on target cells in autoimmune disease [Heufelder et al. 1991; Erkeller-Yüksel et al. 1992;
Table 1.2.4 Major Evidence for Heat shock protein involvement in human autoimmune diseases (SLE is discussed in section 4)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Evidence (pro and contra hsp involvement)</th>
</tr>
</thead>
</table>
| Reactive arthritis    | *Pro*: hsp60-reactive T cells confers disease, epitopes unique for mycobacterial hsp60 amino acids 456-465  
               | *Pro*: hsp-reactive a/b T cells; epitope shared for mycobacterial and mammalian hsp60 AA 370-540  
               | *Pro*: cytolytic T-cell clones from sites of inflammation reactive to myco. and human hsp60  
               | *Contra*: low frequency of hsp60-reactive T cells  
               | *Contra*: synovial fluid cells do not respond to human hsp60 in inflammatory synovitis  |
| Rheumatoid Arthritis  | *Pro*: hsp60-reactive T cells in synovial fluid of early chronic arthritis  
               | *Pro*: hsp60-reactive γ/δ T cells in synovial fluid of chronic arthritis  
               | *Pro*: hsp60-reactive T cells in synovial fluid of juvenile chronic arthritis  
               | *Pro*: B and T cell responses to myco..hsp60 in JCA not adult RA  
               | *Pro*: increased serum levels of hsp60 specific antibodies  
               | *Contra*: no hsp60-reactive T cells in synovial fluid of adult RA  
               | *Contra*: autoantibodies to human hsp infrequent  
               | *Contra*: hsp60-reactive T cells responses similar in peripheral samples from patients and healthy individuals  |
| Multiple Sclerosis    | *Pro*: γ/δ T cells in acute plaques  
               | *Pro*: γ/δ T cells in demyelinated lesions  
               | *Pro*: hsp-reactive spinal fluid in T lymphocytes in MS  
               | *Pro*: T cell responses to hsp70 in MS  
               | *Pro*: hsp70 antibodies to serum and cebrospinal fluid  |
| Kawasaki disease      | *Pro*: increased serum levels of hsp60 antibodies  |
| Psoriasis             | *Pro*: increased serum levels of hsp60 antibodies  |

[Taken from:  
*a*: Gaston et al. 1990; Life et al. 1991; Pope et al. 1992  
*c*: Selmaj et al. 1991; Freeman et al. 1992; Georgopoulos and McFarland 1993;  
*d*: Yokota et al. 1993;  
*e*: Rambukkana et al. 1993]
Table 1.2.5 Evidence for surface expression of hsp-related peptides in humans

<table>
<thead>
<tr>
<th>Potential surface antigen</th>
<th>Cell</th>
<th>Method of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp60</td>
<td>PEERγδ T cell</td>
<td>cytofluorimetry, surface iodination</td>
</tr>
<tr>
<td></td>
<td>leukaemia (^a)</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td></td>
<td>Daudi lymphoma(^b,c)</td>
<td>cytofluorimetry, surface iodination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>immunotoxins</td>
</tr>
<tr>
<td></td>
<td>H9 lymphoma(^b)</td>
<td>cytofluorimetry, immunotoxins</td>
</tr>
<tr>
<td></td>
<td>U937 monocytic line(^b)</td>
<td>cytofluorimetry, immunotoxins</td>
</tr>
<tr>
<td></td>
<td>Oligodendrocytes(^d)</td>
<td>immunofluorescence, cytofluorimetry</td>
</tr>
<tr>
<td></td>
<td>pancreatic carcinoma(^b)</td>
<td>cytofluorimetry, immunotoxins</td>
</tr>
</tbody>
</table>

hsp70

|                           | HL60 cells\(^e\)          | immunofluorescence                   |
|                           | tumour cell lines\(^f\)   | cytofluorimetry, surface iodination, immunopptn |
|                           | retro-ocular fibroblasts\(^g\)| immunofluorescence, surface iodination, immunopptn |

hsp90

|                           | HL60 cells\(^e\)          | immunofluorescence                   |
|                           | tumour cell lines\(^f\)   | cytofluorimetry, surface iodination immunopptn |
|                           | blood monocytes\(^h\)     | cytofluorimetry                       |

Immunopptn=immunoprecipitation

[Taken from: \(^a\)Jarjour et al. 1989; \(^b\)Poccia et al. 1992, \(^c\)Fisch et al. 1990; Kaur et al. 1993; Wand-Württenberger et al. 1991; \(^d\)Freeman et al. 1992, \(^e\)Jarjour et al. 1989, \(^f\)Ferrarini et al. 1992, \(^g\)Heufelder et al. 1992, \(^h\)Erkeller-Yüksel et al. 1992]
Hsp90 surface expression has also been demonstrated in hamster fibroblasts when subjected to infection by herpes simplex virus [La Thangue and Latchman 1988]. HL60 cells stimulated with PMA or heat shock showed surface immunofluorescence with mAbs to hsp70 and hsp90, compared to cells which had not been stimulated or heat shocked and when incubated with unrelated mAbs [Jarjour et al. 1989]. These and other findings suggest that under certain conditions, cells expressing hsps on their surface can become targets for autoreactive antibodies. In addition, evidence has been presented for target cell recognition by γδ T cells via hsp [Fisch et al. 1990; Rajasekar et al. 1990; Kaur et al. 1993]. Specific interactions of γδ T cells with tumour cells can be blocked with anti-hsp60 antibodies [Fisch et al. 1990; Kaur et al. 1993]. In addition, there is some evidence that γδ T cells from patients with multiple sclerosis attack oligodendrocytes that express hsp60 on their cell surface [Freeman et al. 1992].

The question arises of how these cytosolic proteins become expressed on the cell surface if they lack sequences for cell surface translocation. It is possible that anti-hsp antibodies cross-react with structurally similar epitopes on unrelated surface molecules; although several immunoprecipitation experiments suggest that the precipitated surface molecules are indeed hsp. Until sequence data is available neither possibility can be eliminated. Alternatively hsps could be translocated to the cell surface by unknown mechanisms. For example it is possible that because of their ability to bind to a wide variety of molecules, hsp60 and hsp70 could be translocated passively by unrelated cell surface proteins. This hypothesis is supported by the fact that coprecipitation of a 70kD molecule with the hsp60 by rabbit anti-
GroEL sera has recently been described [Kaur et al. 1993]. There is also some circumstantial evidence for peptide-acceptor/translocator function of hsp70 and gp96 in the course of antigen processing through the MHC class II and MHC class I pathway respectively [Srivastava and Heike 1991].

T cells with reactivity to hsps have been detected frequently in normal individuals and naive mice [Fu et al. 1993; Munk et al. 1989; Born et al. 1990; Haregwoin et al. 1989]. Recognition of stressed host cells by hsp-specific T cells would provide an ideal means of immune surveillance because it focuses on a single marker indicative of a variety of cellular stresses. Although both α/β and γ/δ T cells may contribute to immune surveillance, several studies have indicated a bias of γ/δ T cells to hsp. A high percentage of γ/δ T cells develop in a thymus independent manner and therefore evade thymic silencing of T lymphocytes with specificity for autoantigens including hsp [Haas et al. 1992]. The type of antigen recognised by γ/δ T cells is unclear but both conventional recognition of hsp-derived peptides in the context of MHC molecules and direct recognition of cell surface expressed hsp have been proposed although the precise mechanism is unknown [Born et al. 1990; Haregwoin et al. 1989; Jarjour et al. 1989; Kaur et al. 1993; Fu et al. 1993; Fisch et al. 1990].
1.3 Heat Shock Proteins and Cancer

Heat shock protein expression has been studied in several different types of cancer using Western blotting, immunohistochemistry and Northern blotting [Fuller et al. 1994].

The expression of hsp genes 89α, 89β, 70 and ubiquitin were studied in pancreatic carcinoma tissue, control pancreatic tissue and chronic pancreatitis tissue (n=5 in each case). Hsp89α was overexpressed selectively 4-5 fold in pancreatic carcinoma compared to chronic pancreatitis, and the tumour cells were shown to contain the largest amount of hsp89α mRNA. Levels of hsp70 mRNA were increased in pancreatic carcinoma and chronic pancreatitis. Hsp89β and ubiquitin were expressed constitutively at high levels in pancreatic tissue from all three groups [Gress et al. 1994]. The authors postulate that since only hsp89α was detected in pancreatic carcinoma, and the tumour cells were the predominant site for transcription of hsp89α, that overexpression of hsp genes is not solely a nonspecific reaction of pancreatic cells to cellular injury and that hsps may play a role in the pathogenesis of pancreatic cancer.

The constitutive expression of hsp90α, 90β, 70 and 27 was studied in leukaemia cell lines, cells obtained from patients with acute leukaemia and normal blood cells by Northern blotting [Yufu et al. 1992]. Western blotting revealed that the leukaemic cells contained larger amounts of hsp90 than normal peripheral mononuclear cells. The expression of the hsp90α gene was enhanced in the leukemia cell lines and the leukaemic cells from patients (n=10) compared to healthy controls (n=5). In contrast the hsp90β gene could hardly be detected in either the leukaemia cells or normal blood
cells. An increased expression of hsp70 gene was observed in only one patient and half the patients with acute lymphoblastic leukaemia showed overexpression of the hsp27 gene. Thus, it is only the hsp90α gene that is overexpressed in the leukemia cells suggesting transcription of the hsp90α gene may be regulated by cellular proliferation whereas that of the hsp90β gene may be independent of cellular proliferation. Synthesis of hsp70 is increased in mitogen-stimulated human lymphocytes, but in this study, except in one case, no enhancement of hsp70 gene expression was observed in acute leukemia cells [Ferris et al. 1988]. The difference in the results may be due to which cDNA for the hsp70 family is used as a probe. Conflicting results have been reported on the change in the expression of hsp70 gene in mitogen-stimulated human lymphocytes [Ferris et al. 1988; Kaczmarek et al. 1987].

The levels of hsp89α has also been investigated in human breast cancer tissue [Jameel et al. 1992; Jameel et al. 1993]. The authors isolated a cDNA clone, AJ1 by immunoscreening a human breast tumour library with a polyclonal anti-serum raised against breast cancer metastasis membranes. AJ1 showed complete homology with human hsp89α. The level of AJ1 was then studied in human benign breast tissue (n=17), breast cancer (n=143) and various breast cancer cell lines (n=5). All tissues were found to have some expression of AJ1 but there were significantly higher amounts of AJ1 in malignant breast tissue compared to healthy breast tissue. No significant correlation was found between AJ1 expression and menopausal status, ER (estrogen receptor) status, clinical or histological size or tumour grade. However there was significant association between high AJ1 levels and histological node involvement. Short term survival was increased in patients
with low levels of AJ1, up to eleven years. AJ1 was also expressed constitutively in several breast cancer cell lines and also in a 'normal' breast cell line. Heat shock was found to induce AJ1 and the breast cancer cell lines showed increased expression of AJ1 following stimulation with oestrogen. Addition of growth factors also resulted in increased expression of AJ1.

Expression of hsp70 in breast cancer tissue has also been investigated [Ciocca et al. 1993]. Levels of hsp70 were determined using Western blotting in patients with negative axillary lymph nodal status (n=345). Patients whose tumours had high expression of hsp70 had significantly shorter disease-free survival. In patients who had received chemotherapy, hsp70 was the only independent predictor of disease recurrence.

Hsp27 expression was studied in patients with neuroblastoma (n=53) and in 17 neuroblastoma cell lines to investigate the relationship between hsp27 expression, stage of disease and \textit{N}-\textit{myc} copy number [Ungar et al. 1994]. Increased hsp27 expression in neuroblastomas was associated with limited stage disease and inversely correlated with \textit{N}-\textit{myc} gene amplification, a feature known to predict poor clinical outcome. An inverse correlation was also observed between \textit{N}-\textit{myc} gene amplification and hsp27 protein levels among the neuroblastoma cell lines analysed. Immunohistochemical staining of sections of neuroblastomas showed that hsp27 was expressed most prominently in the cytoplasm of large ganglionic tumour cells present in neuronally differentiated areas of the tumours. Interestingly, differentiation of these cell lines using retinoic acid resulted in increased expression of hsp27. Retinoic acid decreases \textit{N}-\textit{myc} expression and cellular proliferation [Thiele et al. 1985]. Thus, it is likely that there may be some interrelationship
between \textit{N-myc} and hsp27 protein levels and differentiation and proliferation status of neuroblastoma cells.

Hsp27 expression has been studied extensively in breast carcinoma, where overexpression of hsp27 has been associated with shorter disease free survival in patients with local disease, although it does not provide prognostic value independent of other indicators such as the presence of disease that has spread to the lymph nodes [Thor \textit{et al.} 1991]. Another study investigated immunohistochemical analysis of tumours in patients (n=361) with primary breast cancer in relation to disease-free survival (DFS), survival from first relapse and estrogen and progesterone receptor status [Love and King 1994]. Patients with tumours positive for hsp27 had a prolonged survival from first relapse but short DFS. This association with short DFS was only true in patients with no nodal involvement and agreed with data published elsewhere [Tandon \textit{et al.} 1991]. Hsp27 has also been shown to predict for hormone sensitivity of advanced breast cancers [Love and King 1994].

There have also been several studies examining hsp27 expression and both drug and multidrug resistance in breast cancer cells [Oesterreich \textit{et al.} 1993; Ciocca \textit{et al.} 1992]. Transfections of breast cancer cells, that usually have low levels of hsp27, with a full length hsp27 construct, resulted in 3-fold elevated resistance to Doxorubicin (a chemotherapeutic drug). When these cells were transfected with an antisense hsp27 construct, they were rendered sensitive to Doxorubicin. Hsp27 expression is associated with improved response to chemotherapy and longer survival in patients with metastatic breast carcinoma [Seymour \textit{et al.} 1990]. In malignant fibrous histocytoma, a common form of sarcoma in adults, hsp27 expression is associated with
longer survival [Tetu et al. 1992]. In addition, patients who developed metastatic disease were more likely to respond to chemotherapy if their tumours expressed hsp27. This is in contrast to the in vitro data which suggests that hsp27 expression is associated with resistance to chemotherapeutic drugs [Ciocca et al. 1992; Huot et al. 1991].

Thus, high levels of hsp27 can be associated with both a good prognosis in some malignancies and a poor prognosis in others. This could indicate that hsp27 has different roles in different tissues or that there are other elements present in some malignancies that can override or bypass the effect of hsp27.

Immunisation with hsp gp96, hsp90 or hsp70 isolated from distinct tumours has been shown to result in specific immune responses against the homologous tumour [Srivastava et al. 1986, Srivastava 1993, Udono and Srivastava 1993]. However, it appears not to be the hsp itself that causes this immune response, rather the peptides that are attached to it. This was demonstrated by the immunisation of mice with either hsp70-derived from MethA sarcoma or hsp70 purified from normal tissue. The tumour diameter in the mice immunised with MethA-derived hsp70 showed a considerable reduction unlike those in mice immunised with normal purified hsp70. However, when the MethA derived hsp70 was purified using ATP-affinity chromatography there was no reduction in the tumour, showing that it is the peptides that are responsible for this immune response rather than the hsp per se.

The identification of hsps as chaperones of antigenic peptides may facilitate the development of the use of immunotherapy in cancer treatment. One of
the main problems in cancer immunotherapy is the likelihood that human and animal cancers are antigenically distinct thus making identification of immunogenic antigens from individual tumours from cancer patients impractical. The observation that hsp60 chaperone such antigenic peptides avoids this problem.
1.4 Systemic Lupus Erythematosus

Epidemiology, Clinical Features, Aetiology

SLE is the most clinically and serologically diverse of the autoimmune connective tissue diseases. It is a disease of unknown aetiology, most commonly affecting young women. A recent report from the United States shows a prevalence rate of between 14.6-50.8 cases per 100,000 people with a sex ratio of women to men of 6-10:1 and a prevalence rate of up to 1 in 250 amongst the black population [Hochberg 1993]. Virtually any organ in the body may be involved and polyarthralgia occurs in almost all patients. The course of the disease is usually a series of relapses and remissions. SLE is characterised by the presence of circulating autoantibodies to DNA, RNA and a host of other cellular antigens. Circulating immune complexes are frequently observed and may be deposited in the kidney, skin, brain, lung and other tissues, causing inflammation and tissue damage. Since SLE is difficult to classify due to its multiorgan involvement various systems have been established based on clinical and immunological data. Table 1.4.3 shows one such classification of this disease.

In the last forty years the highest causes of mortality have been attributable to renal or CNS disease and recurrent infection. However the survival rates of patients with SLE have progressively increased with better treatment. In 1954 the 5 year survival rate was 51% [Merrel and Shulman 1955]; a study in 1989 showed a disease survival rate of 95 % at 5 years and 87% at 10 years [Swaak et al 1989]. The treatment of SLE usually involves combinations of non-steroidal anti-inflammatory drugs (NSAID), anti-malarials, corticosteroids and cytotoxic drugs.
The cause of this disease is unknown but genetic, viral, and environmental factors have all been considered [Mehra et al. 1993, Morrow and Isenberg 1987]. The MHC haplotype A1, B8, DR3 is particularly associated with SLE in Caucasians. Some autoantibodies are linked to HLA markers such as HLA-DR3 (DRw17) and DQw2 with anti-Ro/anti-La antibodies [Arnett 1994, Isenberg and Horsfall 1993]. Studies with monozygotic twins have shown a concordance rate of 70% whilst dizygotic twins have a concordance rate of 15% [Block et al. 1975]; although more recent studies have shown lower concordance figures [Deapen et al. 1992]. Retroviruses have also been considered as a possible trigger factor for SLE [Venables and Brookes 1992].

It is also possible that autoantibodies may arise by molecular mimicry such as occurs between U1 snRNP antibodies and influenza B virus [Guldner et al. 1990]. One interesting piece of evidence that there is an environmental component to SLE came from a study that pet dogs belonging to patients with SLE have increased propensity to develop anti-DNA antibodies [Jones et al. 1992]. Drugs such as hydralazine have also been implicated in triggering SLE but drug induced SLE has several different features to this disease such as: male to female ratio, absence of antibodies against dsDNA and generally absence of renal disease [Tan 1989].

SLE is characterised by the diversity of its immunopathology (table 1.4.1). In SLE the complement system is often involved in inflammatory events. Complement is fixed or consumed by immune complexes and localised in the tissues, particularly in the kidneys. Reduced complement levels are associated with SLE, both the overall haemolytic ability of the complement system is decreased and the levels of most of the major components are all reduced. Cellular abnormalities occur in both the T and B lymphocytes.
Table 1.4.1. Autoantibodies found in SLE and their clinical associations.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen/epitope</th>
<th>Prevalence(%)</th>
<th>Clinical Associations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intracellular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>dsDNA, (ssDNA)</td>
<td>40-90</td>
<td>IgG, present in renal eluates</td>
</tr>
<tr>
<td>Histone</td>
<td>H1,2A,2B,3,4</td>
<td>30-80</td>
<td>Drug-induced SLE +ssDNA</td>
</tr>
<tr>
<td>Sm</td>
<td>A,B/B', D, N</td>
<td>Overall 30-80</td>
<td>Afro-Caribbean, 80% Sm, HLA-DR-2</td>
</tr>
<tr>
<td>U1 RNP</td>
<td>68-kDa RNP</td>
<td>20-35</td>
<td>Mild disease, renal involvement HLA-DR4</td>
</tr>
<tr>
<td>Ro (SS-A)</td>
<td>60,52-kDa protein bound to cytoplasmic RNA</td>
<td>20-30</td>
<td>Rash, congenital heart block HLA DR2 DQw1</td>
</tr>
<tr>
<td>La (SS-B)</td>
<td>47-kDa protein bound to a variety of RNAs</td>
<td>10-15</td>
<td>DR3, congenital heart block</td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>8KDa protein</td>
<td>50-60</td>
<td></td>
</tr>
<tr>
<td><strong>Cell Membrane</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>Phospholipids DNA</td>
<td>20-40</td>
<td>Recurrent abortion</td>
</tr>
<tr>
<td>Neuronal Antigen</td>
<td>expressed on neuronal cell line in vitro</td>
<td>70-90(CNS)</td>
<td>Some crossreact with lymphocyte cell surface</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>T cells&gt;B cells</td>
<td>~70 IgM, ~40 IgG</td>
<td>Cytotoxic Some crossreact with cell surface antigens of CNS.</td>
</tr>
<tr>
<td>Red Cell</td>
<td>Non Rh related</td>
<td>&lt;10</td>
<td>haemolytic anaemia</td>
</tr>
<tr>
<td>Platelet</td>
<td></td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td><strong>Extracellular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheumatoid histones</td>
<td>Fc region of IgG factor</td>
<td>20-30</td>
<td>Usually IgM, crossreact with Ro/SS-A</td>
</tr>
</tbody>
</table>
Cytokine production has also been examined extensively in both patients with SLE and animal models and is summarised in table 1.4.2. A defect often seen is that of IL-2 production and responsiveness. IL-2 seems to play a role in the immunological imbalance of lupus-prone mice. The presence of circulating immune complexes (CIC) is one of the most immunopathological characteristics of SLE. CIC appear to cause damage by depositing mainly in the kidney but also in other tissues. CIC are thought to elicit tissue damage by being deposited in tissues and then fixing and activating complement and recruiting inflammatory cells.

Classification Criteria and Disease Activity Indices

Classification criteria were established by the American Rheumatism Association (table 1.4.3). A patient is considered to have SLE if four or more of 11 criteria are present simultaneously [Tan et al. 1982]. As mentioned previously, patients are subject to flares and remissions in disease activity that can vary greatly over time. Various disease activity indices have been established to assist clinicians in treating patients [Hay 1993]. Since it is also possible that different disease manifestations may have different aetiologies some indices have been devised to separate patients into particular categories based on organ involvement when assessing disease activity. The British Isles Lupus Assessment Group (BILAG) designed disease activity criteria on the physician's intention to treat [Hay et al. 1993] (see Appendix II). This approach assesses disease activity in eight major organs or systems. The BILAG index also makes an allowance for change in activity over time. Each system is assessed individually and scored according to its activity (A-E with A being the most active requiring major immunosuppressive therapy.
Table 1.4.2 Cellular abnormalities and cytokine dysregulation in SLE (adapted from Isenberg and Horsfall 1993)

<table>
<thead>
<tr>
<th>Cell Type/Cytokine</th>
<th>Dysregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes/macrophage</td>
<td>Decr. TNFα production-genetic defect</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Incr. Nos. of activated B cells</td>
</tr>
<tr>
<td>B cells</td>
<td>Hypergammaglobulinaemia</td>
</tr>
<tr>
<td></td>
<td>IgG antibodies reactive with self antigens (cell membrane, cytoplasmic proteins, nuclear antigens, extracellular proteins)</td>
</tr>
<tr>
<td></td>
<td>Incr. IL-2 receptor, decr. CR1 expression, incr. surface expression of hsp90 but not hsp70 compared to normal cells.</td>
</tr>
<tr>
<td>T cells</td>
<td>Decr CD4^+CD45R^+ (subset T helper, suppressor/inducer)</td>
</tr>
<tr>
<td></td>
<td>Incr. CD4^+8^- T cell receptor αβ+ Th</td>
</tr>
<tr>
<td></td>
<td>Activated T cells are class II^+ (DP,DR)</td>
</tr>
<tr>
<td></td>
<td>Defective suppression</td>
</tr>
<tr>
<td></td>
<td>Impaired cytotoxicity</td>
</tr>
<tr>
<td></td>
<td>Activated peripheral T cells</td>
</tr>
</tbody>
</table>

Cytokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Dysregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>Incr. or Decr responsiveness of T cells</td>
</tr>
<tr>
<td>IL-2</td>
<td>Incr. or Decr. Reduction in vitro might reflect transient exhaustion of <em>in vivo</em> activated T cells. IL-2 leads to autoimmunity in thymectomised mice, ameliorates disease in lpr mice.</td>
</tr>
<tr>
<td>IL-4</td>
<td>May have role in B cell hypereactivity.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Elevated in SLE and correlates with disease activity. Reactants induced by IL-6 not elevated in SLE possible abnormality in IL-6 receptor?</td>
</tr>
<tr>
<td>TNFα</td>
<td>MHC-linked production:</td>
</tr>
<tr>
<td></td>
<td>Decr. in HLA-DR2, DQw1 associated with nephritis</td>
</tr>
<tr>
<td></td>
<td>Incr. in HLA-DR3, DR4, no nephritis.</td>
</tr>
<tr>
<td></td>
<td>Accelerates nephritis in animal models</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Incr. in SLE. Augments disease in animal models</td>
</tr>
</tbody>
</table>

TNFα = tumour necrosis factor, IL = interleukin, IFN = interferon and E never having been active in that particular organ system).
Table 1.4.3. Revised Criteria of the American Rheumatism Association for the classification of systemic lupus erythematosus (SLE)

1. Malar rash
2. Discoid rash
3. Photosensitivity
4. Oral Ulcers
5. Arthritis
6. Serositis (i) pleuritis or (ii) pericarditis
7. Renal disorder (i) proteinuria>0.5g/24h or (ii) cellular casts
8. Neurological disorder (i) seizures or (ii) psychosis (after excluding other causes eg drugs)
9. Haematological disorder (i) haemolytic disorder or
   (ii) leucopenia or $4 \times 10^9/l$ on 2 or more occasions
   (iii) lymphopenia or $105 \times 10^9/l$ on 2 or more occasions
   (iv) thromocytopenia $<100 \times 10^9/l$
10. Immunological disorders:
    (i) positive LE cell or
    (ii) raised anti-native DNA antibody or
    (iii) anti-Sm antibody
    (iv) false serologic test for syphilis present for at least six months
11. Antinuclear antibody in raised titre

A person shall be said to have SLE if four or more of the 11 criteria are present, serially or simultaneously, during any interval of observation [Tan et al 1982].
The BILAG index can also be adapted to give a global score using the following score points: A=9 points, B=3, C=1, D=0, E=0. A and B scores are considered to represent active disease in individual systems and a total score greater than 6 implies active disease as a whole. A questionnaire is completed at each clinic visit and is then transferred to a computer for rapid analysis.

Animal Models

Murine strains which spontaneously develop SLE-like syndromes allow us to study changes immediately preceding the development of symptoms and to study closely pathological changes throughout the disease. The murine models of SLE have led to much information being obtained. However, there are some differences in the clinical features of these models and the human disease. All current models show progression of this disease rather than relapses and remissions. In addition, manifestations observed in human SLE are not always present in murine models [Andrews et al. 1978]. In a heterogeneous disease such as SLE, it is possible that some models may represent patients with a particular type of disease. Some of these murine models are also used as other models for other autoimmune disease. Whilst it is useful to follow the disease progression of these mice with disease, SLE patients are exposed to a variety of immunosuppressive treatments and environmental agents which could considerably alter the disease state. Some of the models develop features not seen in the patients with SLE, as in the case of the MRL/lpr mouse which develops massive
Table 1.4.4. Murine Models of SLE (Adapted from Morrow and Isenberg 1987)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean life span (months)</th>
<th>Major Clinical features</th>
<th>Autoantibody specificities</th>
<th>Immunological abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZB</td>
<td>15-18</td>
<td>haemolytic anaemia GN</td>
<td>DNA, RF erythrocyte</td>
<td>Thymic atrophy high IgG/IgM</td>
</tr>
<tr>
<td>NZB/NZW (females)</td>
<td>7-9</td>
<td>severe GN</td>
<td>DNA, RF</td>
<td>Thymic atrophy high IgG/IgM</td>
</tr>
<tr>
<td>MRL-lpr/lpr (females)</td>
<td>3-5</td>
<td>GN, arthritis vasculitis</td>
<td>DNA, Sm RF</td>
<td>Thymic atrophy high IgG/IgM</td>
</tr>
<tr>
<td>BXSB (males)</td>
<td>4-6</td>
<td>GN haemolytic anaemia</td>
<td>DNA erythrocyte</td>
<td>Thymic atrophy high IgG/IgM</td>
</tr>
<tr>
<td>Moth-eaten</td>
<td>1</td>
<td>mild GN hair loss</td>
<td>DNA, RF erythrocyte</td>
<td>Thymic atrophy high IgG/IgM general immunosupp.</td>
</tr>
<tr>
<td>Palmerston North</td>
<td>10-12</td>
<td>polyarthritis GN</td>
<td>DNA</td>
<td>high IgG/IgM</td>
</tr>
<tr>
<td>Swan</td>
<td>18</td>
<td>mild GN</td>
<td>DNA</td>
<td>thymic atrophy</td>
</tr>
</tbody>
</table>

GN glomerular nephritis, RF rheumatoid factor, immunosupp. immunosuppression
lymphadenopathy caused by a defect in the Fas antigen which is involved in apoptosis [Watanabe-Fukunaga et al. 1992]. Neither the massive proliferation of lymphocytes nor a defect in the Fas antigen have been found with patients with SLE [Mysler et al. 1994]. However the models develop anti-dsDNA antibodies in a manner similar to patients with SLE and all develop glomerular nephritis (GN). The ability to manipulate and treat these models has led to much greater knowledge of the aetiology of murine SLE but it is critical to ascertain whether similar processes are occurring in the human disease.

Other Autoimmune Diseases.

Sera from patients with other autoimmune diseases are often used as controls in studies of SLE. It is important to note that there is considerable overlap in autoimmune diseases and that members of the same family can have different autoimmune rheumatic diseases suggesting a genetic link between these diseases.

Rheumatoid Arthritis (RA) is an inflammatory disease affecting primarily synovial joints but it can affect other organs such as the kidneys. It is a chronic autoimmune condition affecting as many as 3% of the population. It is three times more common in women than in men and usually occurs at higher frequency in an older age group. Rheumatoid factors and antinuclear antibodies are present in both diseases but the former tends to predominate in RA and the latter in SLE. The aetiology of RA is uncertain although experimental evidence has suggested that one of several micro-organisms may act as a triggering mechanism, perhaps in association with a genetic
predisposition. Both HLA-Dw4 and HLA-DR4 have been associated with the
disease but it is likely that other factors such as environment and hormones
contribute to this disease. There is some evidence that a specific immune
response against collagen type II is present in patients with RA suggesting a
possible aetiological role.

Sjögren’s syndrome (SS) is a chronic inflammatory disorder of the exocrine
glands manifested by dry eyes and a dry mouth. SS can be a primary event
or occur as a secondary event to other autoimmune diseases. Primary SS
has several features in common with SLE: the same F:M ratio, the same
associated haplotype (HLA A1, B8, Dr3), and a predisposition to fatigue and
arthralgia. A narrower range of autoantibodies is observed in SS with the
most common being against Ro and La antigens.

Scleroderma is characterised by progressive hardening of the skin. There
can also be multi-organ involvement whereby increased collagen production
can occur in organs such as the kidney and lung. This disease also shows
the same F:M ratio as SLE. Antibodies to an antigen Scl-70, identified as
topoisomerase 1 are characteristic of this disease but may occur in only 25%
of patients.

Myositis is a chronic progressive condition characterised by inflammation of
the muscles of unknown cause which probably has multiple aetiologies.
When the disease is accompanied by the presence of a characteristic skin
rash the term dermatomyositis is used. Patients with this disease are more
commonly female (3:1, F:M), and often carry the HLA-B8/HLA-DR3 antigens.
A high proportion of these patients have anti-nuclear antibodies (up to 95%),
to a 56kDa nuclear antigen (85%) and to the Jo-1 antigen (30%).
Juvenile Autoimmune Diseases

There is no specific age below which an autoimmune disease is defined as 'juvenile' with some authors using 16 years, some using 18 years and others 20 years.

Juvenile Systemic Lupus Erythematosus

SLE is rare in children under five with the average onset of symptoms being around 12 years. SLE in children differs from that in adults in several respects. For example, the sex ratio amongst children shows a much less marked predilection for females (4:1 compared to 9:1 F:M), although onset of SLE below the age of 10 shows similar frequency of occurrence between males and females. Studies have shown that childhood cases generally have a more severe onset, with major haematological disease and several autoantibodies, to DNA, Sm and RNP, being more frequent, particularly in the younger age group. Cardiopulmonary disease and Sjögren’s syndrome are significantly less common. There is also a strong association with HLA DR2 and DR3 in these patients, with HLA-DR3 being associated with older onset lupus and HLA DR2 occurring at a higher frequency with younger age onset. The mortality rate has improved with the latest figure being reported as 15% after 10 years with SLE. Infection and renal disease are the major factors in two-thirds of deaths, similar to the adult disease.

Juvenile Rheumatoid Arthritis

Rheumatoid arthritis occurring below the age of 16 is termed juvenile arthritis (JRA). JRA encompasses several different disorders, of which the 10% who
are persistently seropositive for IgM rheumatoid factor most closely resemble their adult counterparts. The frequency of ANA antibodies in seropositive RA and JRA are similar. The major distinguishing features between adult-onset and childhood-onset are the relative frequency of uveitis, pericarditis and amyloidosis, with the common absence of rheumatoid factor in the latter. The use of corticosteroids is generally restricted, because of their interference with growth. These children are predominantly female, over the age of 10 at onset, and with severe polyarthritis in the hands, feet and even hip joints within six months of onset of symptoms.

Juvenile Dermatomyositis

Inflammatory muscle disease in children is more commonly manifested as dermatomyositis (JDM), in contrast to adults where polymyositis is more common, with vasculitis as a predominant pathological feature. The peak age of onset is at around eight years, and there is less female predominance of cases than is found in other autoimmune diseases. The characteristic rash found in children is similar to that found in adults. The mortality rate is much lower in children than in adults, mainly due to the difference in the incidence of cardiac disease and malignancy. There is also greater overlap of JDM with other autoimmune diseases compared to the adult onset of this disease.
1.4 Heat Shock Proteins and SLE

There are three main areas of published work relevant to the research presented in this thesis. These are studies that describe overexpression of hsps in SLE, the detection of antibodies to hsps in SLE, and the surface expression of hsps in peripheral blood mononuclear cells in SLE.

Over expression of hsps in SLE

In 1987 it was demonstrated that increased synthesis of two proteins occurred in pooled peripheral blood mononuclear cells (PBMCs) from 20 patients with SLE, none of whom were described as having a fever compared to healthy age and sex-matched volunteers. These proteins had molecular weights of 70kDa and 90kDa. This increase was shown to be at the transcription level by completely blocking it with the transcriptional inhibitor actinomycin D [Deguchi et al. 1987]. More recently it was demonstrated that PBMCs taken from five patients with SLE had spontaneously higher rates of transcription of the hsp70 gene compared to healthy controls; three asthmatic patients taking high doses of steroids did not have higher rates of transcription of the hsp70 gene [Deguchi and Kishimoto 1990]. The method used here was a nuclear run on assay, where the amount of radiolabelled nucleotide incorporated into nascent RNA transcripts is measured directly, and detected by hybridisation to an hsp70 cDNA probe.

Hsp90 levels were quantitated in PBMCs of individual patients with SLE, in this laboratory. Intially this was developed to investigate the role of hsp90 in
the sensitivity of cells to steroid treatment [Norton et al. 1989]. The method employed used a monoclonal antibody to hsp90 (courtesy of Professor David Toft). However since this antibody can recognise up to four proteins of MW 35-40 kDa it was unsuitable for use in an ELISA assay. Therefore quantitation was achieved by Western blotting and scanning densitometry. The levels of hsp90 in PBMCs of 155 of the SLE patients (n=57) were found to be elevated compared to age and sex matched healthy controls (n=12). The authors found no correlation between raised protein levels and fever or steroidal treatment. A disease control group of patients with RA was also included (n=12) and none of these patients had elevated levels of hsp90. The highest levels of hsp90 were found in patients with active disease, although not all patients with active disease had high levels of hsp90. In one patient, high hsp90 levels were found to decrease following plasma exchange and a reduction in disease activity.

Further studies investigated the relationship of hsps at the protein level in SLE as well as a variety of other autoimmune disease [Dhillon et al. 1992; 1993]. Studies of PBMC samples from SLE patients (n=102) and samples from normal controls (n=59) demonstrated convincingly that hsp90 levels are elevated in approximately 30% of SLE patients. Moreover, this elevation is found primarily in patients with active disease particularly involving neurological or cardiothoracic systems. This elevation is largely restricted to SLE patients compared to patients with other autoimmune diseases. No alteration in expression levels of hsp65 or hsp73 was observed but increased expression of hsp72 was detected in 9% of patients with SLE. Levels of hsp72 and hsp90 do not correlate in individual patients. Hsp90 levels are independent of the presence of fever or other disease
markers such as erythrocyte sedimentary rate (ESR) or anti-DNA antibodies. These results suggest that overexpression of hsp90 in SLE patients is not simply a generalised stress response.

Transcription of hsp genes in SLE has also been studied in this laboratory [Twomey et al. 1992, 1993]. Transcription rates of the hsp90β gene but not the hsp90α gene are significantly elevated (20%) in several SLE PBMC samples (n=20), compared with age and sex-matched controls (6%) (n=16). Moreover, elevated hsp90β gene transcription, but not hsp90α, correlates with significantly elevated hsp90 protein in these samples. Increased transcription of the hsp70 gene has also been observed in SLE patients (n=30) compared to healthy controls (n=30) but this was not found in the same patients with elevated transcription of the hsp90β gene [Twomey et al. 1993a]. In addition, there was no correlation between transcription of the hsp70 gene and hsp72 protein levels; nor was there over expression of ubiquitin genes in SLE patients compared to healthy controls [Twomey et al. 1993b]. No correlation was found between transcription of the hsp90α, hsp90β, hsp70 or ubiquitin genes.

Elevated transcription of the hsp70 gene has been reported in PBMCs of patients with active SLE, five times greater than healthy controls, although the sample size of each group was very small [Deguchi and Kishimoto 1990]. Enhanced transcription of hsp70 has also been observed in murine models of SLE. Northern blotting and nuclear run on assays were used to investigate the level of transcription of an hsp70 gene in the kidney lymphoid cells of MRL/+/pr- lpr mice [Deguchi 1991]. It was found that there was a ten fold greater transcription rate in these cells compared to those from control (MRL/++) mice (which develop very mild disease at a much older age). This
elevation appeared to correlate with a deterioration in renal function and with development of renal lesions in lupus-prone mice.

Previous work in this laboratory has also demonstrated the elevation of hsp90 and hsp72 but not hsp73 or hsp60 in the MRL/lpr-lpr mice compared to the control Balb/c mice or MRL/++ mice but not in any other tissue studied [Faulds et al. 1993]. Elevation of hsp90 levels in MRL/lpr-lpr mice occurs prior to the onset of overt clinical or serological disease (anti-DNA antibodies) at two months of age. There appear to be different expression patterns for hsp90 and hsp72. Hsp90 is elevated in the spleen but not in any other tissues studied, whereas hsp72 is raised in several tissues including heart, brain, kidney and liver.

Autoantibodies to hsp73 in SLE

Sera from SLE patients (n=34) were tested for antibodies to hsp73 [Minota et al. 1988a]. IgM autoantibodies were present in 38% of patients and 15% had IgG autoantibodies. No data concerning age, sex, disease activity, or treatment of these patients was provided. These antibodies were detected by immunoblotting of sera and solid-phase immunoprecipitation using hsp73 specific mAbs. These antibodies were also detected in two out of 16 patients with childhood SLE, but were not found in patients with RA, ankylosing spondylitis, acute hepatitis or in healthy volunteers (n=10).

The same group also described the presence of autoantibodies to hsp90 in 47% (n=15) patients and 33% of patients with myositis (n=6), but not in healthy controls (n=10), RA sera (n=10) nor scleroderma (n=7) [Minota et al. 1988b]. This data was achieved by SDS PAGE followed by Western blotting
with the source of hsp90 being human T cell lysates. These autoantibodies were exclusively of the IgG isotype. These autoantibodies were shown to bind specifically to hsp90 by removing hsp90 from cell lysates by immunoabsorption with a specific anti-mouse hsp90 and by demonstrating increased synthesis of the reactive protein following heat-shock of these cell lysates.

Subsequently, however, these authors reported rather different results using a similar method of immunoblotting, with human cell lysate extracts as source of antigen and positive controls being polyclonal rabbit anti-sera to hsp60 and hsp90 and mAb N27 to unspecified members of the hsp70 family [Jarjour et al. 1991]. They describe a much lower frequency of autoantibodies: one of 48 SLE patients had IgG anti-hsp90 antibodies and six anti-hsp73 antibodies; no RA patients had anti-hsp90 antibodies and only one RA patient had hsp73 antibody. With myositis patients, the only antibody detected was to hsp60 (GroEl). In the healthy controls 3% had anti-73 and anti-hsp60 antibodies, anti-hsp90 antibodies of either isotype were not detected. Anti-hsp60 antibodies were detected more frequently (20% sera were positive) in mixed tissue connective diseases, myositis, Crohn's disease and various autoimmune skin diseases. Anti-hsp73 antibodies were found to be more frequent (20% of sera were positive) in Lyme disease and ulcerative colitis.

Thus there are several differences in these experiments undertaken by the same group. The assay was difficult to standardise, especially since the authors reported that sensitivity varied greatly with incubation of blots and therefore selected an arbitrary time point for incubation with sera assuming that a normal control serum would be unlikely to have anti-hsp antibodies.
The authors fail to mention how many control sera they selected to establish these conditions. The fact that the authors had to retract their original data confirmed this and the authors also stated that they might only be detecting a very small proportion of these antibodies since they were unsure as to what incubation time to use. The sera was treated differently in both sets of experiments; in the former they were heated for a considerable length of time which may affect the stability of the antibody. The methods used did not allow quantitation of antibodies.

More recently a link between antibodies to the constitutive bovine and human 73kDa hsp (but not the inducible 72kDa isoform) in mixed connective tissue disease (MCTD) has been described [Mairesse et al. 1993]. In a study using bovine hsp73 in its native form as the antigen in an ELISA. Elevated levels of IgG anti-hsp73 antibodies were found in patients with MCTD, RA, and scleroderma but not in patients with SLE, dermatomyositis nor healthy controls. The difference between these results and other studies may be due to the use of purified antigen in its native form in a more sensitive assay system. One could argue that human antigen is a more suitable antigen than a bovine one. This enabled accurate quantitation of this antibody leading to the conclusion that both the frequency of the antibody and the mean level were much higher in patients with MCTD. The authors speculate that the very high mean level of these antibodies in MCTD patients may be indicative of the pathogenesis of the disease and have possible application as part of a diagnosis procedure for this disease, although they do not propose a mechanism of action.

The prevalence of antibodies to hsp60 and hsp70 in patients with SLE has also been investigated [Tsoulfa et al. 1989]. The purpose of this study was to
determine whether reactivity to mycobacterial hsp60 plays a significant role in the pathogenesis of RA. Antibody levels to a variety of mycobacterial antigens as well as recombinant hsp60 and hsp70, *E. coli* hsp60 and hsp70, and human hsp70 were studied in patients with RA (n=90), SLE (n=18), healthy controls, ankylosing spondylitis, and TB. The authors found that over 75% of SLE patients (n=18) had elevated levels of IgA antibody to recombinant mycobacterial hsp70 and over 70% had elevated IgA levels to recombinant human hsp70. Levels of IgG antibodies to recombinant mycobacterial hsp70 and *E. coli* hsp70 were also raised in the SLE group but to a lesser degree. In contrast levels of IgM antibodies were not significantly different from the control group.

Another group investigating the role of anti-hsp antibodies in patients with RA (n=99) and SLE (n=48) found several differences in these patients compared to healthy controls (n=65) [Panchapakesan *et al.* 1992]. The authors found increased levels of IgM antibodies to mycobacterial hsp65 in both patients with SLE and RA compared to aged and sex matched controls but no increase in IgG antibodies to the mycobacterial hsp65. Sera were not tested for IgA antibodies. These results differed to those of Tsoulfa and colleagues who found elevated IgA but not IgM antibodies to the mycobacterial hsp65 [Tsoulfa *et al.* 1989]. Panchapakesan and colleagues, found that binding to anti-hsp65 was paralleled by IgM anti-BCG responses. It has been thought that the 65kDa protein is a dominant antigen in BCG and other mycobacteria [Young *et al.* 1991]. Therefore it is possible that these antibodies reflect exposure to mycobacteria, possibly environmental, since the BCG vaccination is not nearly as commonly used in Australia (where Panchapakesan's group is based), compared to the UK (where Tsoulfa is
based). The only difference in antibodies to hsp70 were in RA patients and were of the IgG isotype, and these antibodies were not elevated in SLE patients or the healthy controls. Tsoulfa and colleagues concluded that there was something particular about the IgG response to the mycobacterial antigen in RA when compared to healthy controls or the anti-hsp70 response. However Panchapakesan and colleagues disagree with this view as has Winfield and colleagues [Jarjour et al. 1991]. It is unlikely that the difference would be due to technical variation between the two studies, since both used identical antigens and a similar assay system. However, the antibodies are detected in different groups of patients. Panchapakesan and colleagues, were also unable to reproduce the work of Tsoulfa showing that elevated levels of IgA antibodies are present in patients with RA albeit in a preliminary study and also concluded that it was unlikely that antibodies to these hsps to have a critical role in the development of autoimmune disease.

An Austrian group investigated the presence of antibodies to hsp70 in SLE patients (n=47) and age and sex-matched healthy controls (n=47) [Kindás-Mügge et al. 1993]. The authors found similar frequencies of antibodies recognising hsp72 and/or hsp73 in healthy controls and SLE patients; IgG antibodies were detected in 23% of healthy subjects and 21% of patients with SLE and the frequency of IgM antibodies was 35% and 30% respectively. Western blotting was used to detect antibodies recognising denatured antigen. The authors attempted to quantitate their results by selecting sera with the highest reactivity (i.e. those sera giving rise to the darkest bands) and titrating these sera out. Three sera with high IgG and IgM activity were selected from each group. Using this approach, titres for IgG antibodies (to both hsp72 and hsp73) were found to be comparable between
the two groups, but IgM antibodies (to both hsp72 and hsp73) were much higher in the SLE group. No correlation between the presence or titre of anti-hsp antibodies with clinical or serological parameters was found. Again this group showed discrepancies in their data compared to other groups although the data for antibodies to hsp73 agreed with that of Minota [Tsoulfa et al. 1989; Jarjour et al. 1991; Panchapakesan et al. 1992; Minota et al. 1988]. It is possible that the use of purified antigens may have resulted in a more sensitive assay or that the antigenicity of the hsp was affected by purification, particularly on the immunoblots. This might be caused by slight differences in the tertiary structures or by interaction with other proteins co-migrating with the hsp. It is also likely that long incubation with sera may favour the binding of low affinity antibodies which might be responsible for discrepancies in the two sets of Western data [Jarjour et al. 1991; Kindás-Mügge et al. 1993]. Jarjour and colleagues observed staining by normal sera when they incubated the blots for more than 4h at low temperature. Therefore they chose assay conditions where normal sera did not give a positive reaction with hsp73. However, most of the sera from patients with various autoimmune diseases, including SLE, were also non-reactive under these conditions, which was in contrast to earlier findings reported by the same laboratory [Minota et al. 1986]. In contrast Kindás-Mügge and colleagues used a higher serum dilution, longer incubations and lower temperature. The authors suggested that these antibodies to hsp72 and hsp73 are probably naturally occurring and therefore unlikely to play a major role in SLE.

Muller and colleagues developed an ELISA using commercially available purified ubiquitin, as well as a synthetic fragment of the protein. Ubiquitin
was also used in immunoblotting experiments [Müller et al. 1988]. This group demonstrated that nearly 80% of SLE patients (n=161), had anti-ubiquitin antibodies whereas only 55% of them possessed anti-dsDNA antibodies. 74% of these patients had antibodies to the synthetic ubiquitin peptide fragment; 3% and 5% healthy controls (n=87) had antibodies to ubiquitin and to the synthetic ubiquitin fragment respectively. Disease control groups were also studied including scleroderma, juvenile chronic arthritis, and Sjögren's syndrome, with 16% or less of sera in these groups having autoantibodies, the percentage tending to diminish as the size of the group studied increased. This group then undertook a detailed longitudinal analysis of several autoantibodies in patients with SLE, RA and tuberculosis; and found that 58% of SLE patients (n=12) had raised levels of antibodies to ubiquitin (greater than mean plus 2SD of 50 healthy controls) [Müller et al. 1990]. Pooled results from these patients indicated that disease activity did not appear to correlate with anti-ubiquitin antibodies. However, correlation with clinical features indicated that patients with renal activity were least likely to have these antibodies, although histone and ubiquitin have been detected by immunofluorescence in immune complex deposits in human and murine lupus nephritis biopsies [Batsford et al. 1992]. In addition, analysis of serial bleeds from individual patients showed that high levels of anti-ubiquitin antibodies often correlated inversely with active SLE and anti-dsDNA antibody levels in individual patients.

In summary the data regarding the presence and level of autoantibodies to the hsp90 in SLE and how these relate to disease activity, and other autoantibodies is conflicting. The difficulty of obtaining highly purified antigen is one reason for the variety of data published.
Surface expression of hsps in SLE

A 90kDa protein was shown to be a lymphocyte surface membrane target of anti-lymphocyte antibodies found in SLE [Minota and Winfield 1987]. This protein was later identified as hsp90 and has been shown to co-purify with inner surface of membranes. Immunofluorescence however, has shown that hsp90 remains intracytoplasmic [Koyasu et al. 1986].

Surface expression of hsp90 or some component of it has been detected on the surface of lymphocytes and monocytes of patients with SLE (n=62) compared to healthy controls (n=25) using a mAb AC88 in fluorescence activated cell sorting (FACS) analysis [Erkeller-Yuksel et al. 1992]. The authors suggested that there appeared to be an heterogeneous pattern of surface hsp90 in different cell types occurring in monocytes, B cells, CD3+ T cells and CD4+ but not CD8+ T cells. No surface expression was detected using mAb 4B9/89 in SLE patients which is an antibody directed against hsp60 using the same procedure. Hsp90 protein levels were quantitated in in the peripheral blood mononuclear cells of some SLE patients using Western blotting where surface expression had been examined. Samples where surface expression of hsp90 was detected showed correlation with high levels of hsp90 [Erkeller-Yuksel et al. 1992].

Thus there is evidence of overexpression of hsp90 at the protein and transcriptional level in SLE as well as surface expression of hsp90 in the PBMCs of the same SLE patients. Thus the increased levels of hsp90 in some of the patients with SLE may result in a proportion of the protein, or
some component of it becoming localised to the cell surface. It is possible that hsp90 might arise on the cell surface by chaperoning other proteins or as a direct involvement of hsp90 in antigen presentation. This surface expression could then result in the production of autoantibodies to hsp90.

Therefore the initial aim of this thesis was to devise a suitable method for detecting the presence of autoantibodies to hsp90 in patients with SLE and quantitate these levels. Sera from patients with other rheumatic diseases was also tested, to determine whether these antibodies are limited to patients with SLE, or common to patients with a variety of other autoimmune diseases. In order to establish whether these antibodies are part of a generalised anti-hsp response, a suitable method for detecting antibodies to hsp70 was also established.
Chapter 2 Patients, Materials and Methods

2.1 Patients

1. Patients with Autoimmune disease

All SLE patients attending the Bloomsbury Rheumatology Unit fulfilled the revised criteria of the American College of Rheumatology for the classification of the disease [Tan et al. 1982]. SLE patients were assessed clinically using the BILAG (British Isles Lupus Assessment Group) system whereby each organ or system: general, renal, musculoskeletal, central nervous, mucocutaneous, haematological and cardiovascular/respiratory is scored according to its activity (A-E, with A the most active requiring major immunosuppressive therapy and E never previously active) [Hay et al. 1993]. This system is based on the principle of the physician’s intention to treat (Appendix II). In this study the patients were deemed to have active disease in a given system if they had an A or B score on the date the blood was taken. Categories C, D or E were regarded as inactive. A global score can be determined using the BILAG system by allotting an A category 9 points, B 3 points, C 1 point and D/E no points. Patients with a global score above six were considered to have moderately or severely active disease. This data has been entered onto a database run on an Apple Macintosh Computer as part of ongoing research projects thus enabling easy access to clinical information. HLA data on these patients were also documented. This information was provided by Professor R. Batchelor (Hammersmith Hospital) obtained as described by Fielder et al. (1983). Routine DNA binding, ESR levels, C3 levels and rheumatoid factor status (RF) were determined by the Middlesex Hospital routine laboratories.
As disease controls, patients with myositis (n=18) who met the criteria of Bohan and Peter [Bohan and Peter 1975] were studied, as were patients with primary Sjögren's syndrome (n=18) classified according to the criteria of Isenberg et al. (1984), patients with RA (n=15) classified according to the revised ARA criteria (Arnett et al. 1988), patients with scleroderma (n=17) as defined by Masi et al. (1981), and patients with osteoarthritis diagnosed on clinical and radiological grounds.

Healthy Controls
Sera from 28 healthy individuals (27F 1M) were obtained from age and sex matched controls (age ranged 23-51 years). Additional sets of sera was obtained from 15 healthy non-Caucasians (12F 3M).

Juvenile Autoimmune Diseases
Sera were collected from children with juvenile onset systemic lupus erythematosus, each of whom met four or more of the revised classification criteria of the American College of Rheumatology [Tan et al. 1982]; patients with juvenile chronic arthritis (JCA), (seven with systemic onset, 29 with pauci-articular onset; 17 with sero-negative polyarticular disease, two with sero-positive disease) diagnosed according to the revised criteria of the American College of Rheumatology and patients with juvenile dermatomyositis diagnosed according to the criteria of Bohan and Peter. Sera from 19 children with non-autoimmune conditions (eg. hypermobility, idiopathic limb pains of childhood and diffuse or localised pain syndrome) were used to establish a normal paediatric range in the assays used. All of these sera were obtained during routine clinic visits to the Paediatric Rheumatology Clinic at New England Medical Center, Boston. In addition
synovial fluid was tested from juvenile patients with rheumatoid arthritis. These samples were provided by Dr. L. Tucker Paediatric Rheumatology Clinic at New England Medical Center, Boston.

Since none of the published lupus activity indices have been validated for use in paediatric onset cases a ‘formal’ activity score was not utilised in these patients. However, the case notes of the patients with lupus were reviewed and the relevant clinical and serological data were determined. As for childhood onset lupus, no standard disease activity index exists for children with JCA. In this study, a physician’s global disease activity scale designed for use in outcome assessment in JCA was utilised. Disease activity at the time at the time sera was taken was recorded by physician as R (or 0) = remission (no visible signs of active disease, no systemic manifestation and off all medications); 1 = mild activity; 2 = moderate activity; 3 = severe activity. Disease activity was rated by global assessment of the amount of joint swelling, limitation of motion, heat, pain or tenderness, presence of systemic rash and/or fever, or presence of other extra-articular problems such as pericarditis.

**Preparation of Sera**
Fresh venous blood was collected in additive-free tubes and allowed to clot for 1h at room temperature and centrifuged for 10 min. The upper layer was then centrifuged in eppendorfs tubes for 5 min. The upper layer was then removed and frozen at -20°C until required.

**Patients with Cancer**

Sera samples from patients with malignant and benign breast tumours were provided provided by the Oncology Department at Charing Cross Hospital
London (courtesy of Dr. Y. Luqmani). Clinical data was provided after antibody levels had been quantified, courtesy of Gill Brünstrom. Sera from patients with small cell lung carcinoma was provided from Dr. Jonathan Lederman at Middlesex hospital. All sera was stored at -70°C until required.

2.2 Monoclonal Antibodies

Mouse M.Ab AC88 was a gift from Professor D.O.Toft, Mayo Clinic Foundation Rochester, Minnesota USA. AC88 was raised in mice and is of subclass IgG1. It was developed following isolation of hsp90 from the watermould *Achyla ambisexualis* and binds to human hsp90. [Riehl et al. 1985]. Rat M.Ab AC16 was also a gift from Professor D.O.Toft. and binds to native hsp90 with greater affinity than to denatured hsp90. The antibody to hsp70, which recognises primarily hsp73 but also hsp72 was obtained from Stressgen, Victoria, Canada and used as directed. D5 a monoclonal antibody against hsp27 was a gift from Professor Roger King, Breast biology group, School of Biological Sciences, University of Surrey Guildford, U.K.

2.3 Preparation of Protein

Purified hsp90 was initially found to be labile. Therefore after reconstituting the protein in sterile phosphate buffered saline (PBS) to a concentration of 1mg/ml it was separated into 10ul amounts and stored at -70°C until needed. Each time a new batch was prepared; one aliquot was run on a Coomassie gel to check that there was only one discrete band and another aliquot was Western blotted to ensure its recognition by AC88 and AC16. Similar procedures were also performed with other hsps.
2.4 Direct ELISA for anti-hsp90 antibodies

The method selected to detect antibodies to hsp90 was by ELISA. This was chosen for several reasons, there was limited amounts of sera available from the majority of patients, thus Western blotting was not a viable option. In addition ELISA offers several advantages in that it allows rapid screening, less antigen and sera are required and ELISAs are much more sensitive [Kerney and Challacombe 1988]. Several ELISAs had been described for detecting antibodies to hsp60 and ubiquitin [Stevens et al. 1992; Tsoulfa et al. 1988,1989]. These conditions were tried initially but not found to be very satisfactory. In particular blocking with BSA appeared to block all binding sites available and when the percentage of BSA was increased the antibody response was dampened. Other blocking reagents were then tested and none were found to be satisfactory. However rather than blocking on the plate directly, if the sera were pre-incubated with BSA and goat sera this reduced the background binding but did not dampen the antibody response to the antigen. Checkerboard ELISAs, where the concentration of antigen and antibody were titrated out against each other were developed to optimise the concentration of antigen and sera used for this ELISA. Table 2.1 shows the various conditions tried in the development of an ELISA to detect antibodies to the purified hsp90. The successful method is detailed below.

Nunc ELISA (Dynatech) plates were coated with 100μl per well of purified hsp90 at 0.5μg/ml (Stressgen, Canada) overnight (4°C) in 0.05M bicarbonate buffer pH9.6 (BIC). Half of each plate was coated only with 0.05M bicarbonate buffer. Initially this ELISA was established using AC16, a monoclonal antibody to hsp90 as a positive control (a gift from David Toft).
Table 2.1 Conditions used to develop suitable ELISAs to detect antibodies to hsp90 and hsp70

**Coating Conditions**

i) Antigen: concentration of 0.25-10ug/ml in BIC for 1-12h at 4°C, RT, 37°C  
ii) Antigen: concentration of 0.25-10ug/ml in PBS for 1-12h at 4°C, RT, 37°C

**Blocking Conditions**

i) Blocking using 0.5-10% BSA for 1-4h at 4°C, RT, 37°C  
ii) Blocking using 0.5-10% Casein for 1-4h at 4°C, RT, 37°C  
iii) Blocking using 0.5-10% Marvel for 1-4h at 4°C, RT, 37°C  
iv) Blocking using 0.5-10% Gelatine for 1-4h at 4°C, RT, 37°C  
v) No Blocking

**Washing Conditions**

i) PBS-Tween 20  
ii) PBS  
iii) TBS  
iv) TBS-Tween

**Sera Dilution**

Incubation times varied from 1-12 h at 4°C, RT, and 37°C  
i) Sera diluted from 1:25 - 1:2000 in PBS  
ii) Sera diluted from 1:25 - 1:2000 in PBS-Tween  
iii) Sera diluted from 1:25 - 1:2000 in PBS-Tween and 1% goat serum  
iv) Sera diluted from 1:25 - 1:2000 in PBS-Tween and 1% goat serum and 1% BSA

**Conjugate**

Incubation times varied from 1-12 h at 4°C, RT, and 37°C  
i) F(ab')2 and whole molecules conjugated to either alkaline phosphatase or horseradish peroxidase were used according to manufacturers directions.

The plates were read on a Dynatech MR4000 ELISA reader.
Sera were diluted to 1:200 in filtered (0.45mm) phosphate buffered saline (PBS) and polyoxyethane sorbitan monolaurate: Tween20 (PBS-T) 1% BSA and 1% goat serum (Seralab, UK). Sera were added to the ELISA plate in duplicate wells for 1h at 37°C. After five washes with PBS-Tween, 100μl anti-human IgG or IgM F(ab')₂ alkaline phosphatase conjugated antibodies (Sigma, UK) at 1:50 000 and 1:2000 respectively, in PBS-Tween containing 1% BSA and 1% goat serum was added. This was incubated at 37°C for 1hr. After six washes with PBS-Tween followed by 2 washes with bicarbonate buffer. The colour was developed using p-nitrophenyl phosphate tablets (5mg, Sigma) as substrate. Two tablets were dissolved in 10ml of BIC buffer to which 20μl of 1M MgCl₂ had been added. The reaction was allowed to develop at 37°C. Plates were then read on a Dynatech 4000 ELISA reader (Dynatech, Billinghurst, UK.) at 405nm with reference filter 490nm.

OD values obtained from the wells with no antigen were subtracted from the antigen coated wells and the binding of each individual serum was expressed as a percentage of a positive control sera applied in duplicate on each plate. As further control a known negative serum was applied to each plate. Patients were considered to be autoantibody positive if the corrected OD value was greater than the mean +2SD of 28 healthy controls. Curves showing the binding of sera to hsp90 is shown in fig 2.1. Any sera that gave values outside the linear range were not included in the results.

Inhibition ELISA

This ELISA was performed to demonstrate the specificity of the antigen, by showing that the binding of the antibody to the antigen can be inhibited by
pre-incubation with the antigen coated on the plate. This was carried out on a small selection of sera positive for the antibody being tested and the monoclonal antibody, using the protocol described previously for detecting antibodies to hsp90, except that the test sera, positive and negative control antibodies were pre-incubated with purified hsp90 at a concentration of 10μg/ml at room temperature for 1hr. This mixture was titrated across the plate at three fold dilutions from 1:50. The ELISAs were completed as described for the direct ELISAs.

Anti-hsp70 ELISA

This ELISA was performed as described previously for hsp90 with initial ELISAs being titrated out in a checkerboard pattern. Purified antigen was a mixture of hsp72 and hsp73 with greater amounts of hsp73 being present. The optimum conditions for this ELISA were obtained when the concentration of antigen was 1μg/ml and by leaving the conjugate on the plates overnight at 4°C. A monoclonal anti-hsp70 antibody (Stressgen) was used to initially establish optimum conditions as the positive control. The remainder of the ELISA was identical to that for hsp90. Inhibition ELISAs were performed as for anti-hsp90 antibodies.
Fig 2.1 Dose dependent curve of positive sera binding to human hsp90 by ELISA. Sera was titrated from a concentration of 1:50 across the plate.

2.5. Protein Electrophoresis and Western blotting

The test protein was suspended in a sample buffer (Appendix I). Samples were boiled for 5 min. and stored at -20°C until required. Prior to use the samples were boiled for 5 min.

Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli [1970] using a vertical one dimensional electrophoresis system was used (Gibco-BRL, Paisley, Scotland). Proteins were loaded and electrophoresed in duplicate on two halves of the same gel, in running buffer for 1.5 hr. at 30mA through the stacking gel and 2.5 hr., at 45mA through the lower gel at room temperature.
Half of each gel was stained with Coomassie blue (Sigma, Dorset U.K.) and destained, the other half underwent Western blotting onto nitrocellulose Hybond C (Amersham, Buckinghamshire UK); using tank blotting apparatus (Gibco-BRL), in a circulating buffer overnight at 210mA.

Development of Western blots using monoclonal antibodies

Western blots to be incubated with a particular monoclonal antibody were blocked with blocking buffer for 1hr at room temperature and and washed with washing buffer (WB) three times for ten minutes each at room temperature and once in TBS for 5 min. AC88 was diluted in WB to a concentration of 10µg/ml and incubated with membranes for 2hrs at room temperature. Anti-hsp72/73 antibody was used at the same concentration. After three washes in WB and once in TBS as described above blots were then incubated with anti-mouse immunoglobulins (raised in rabbits) conjugated to horseradish peroxidase (Dakopatts, Glostrup, Denmark) at a concentration of 1 in 300 dilution in WB. Blots were then developed using enhanced chemiluminescence (ECL) kit (Amersham UK), with multiple autoradiograph with exposures of 5-40s.

2.6. Production of Monoclonal Antibodies

Since it was possible to detect hsp90 antibodies in patients with SLE, an attempt was made to generate human immunoglobulin anti-hsp antibodies from several patients with SLE. The spleen has been shown to be an excellent source of lymphocytes for fusions but in the absence of available spleen tissue PBLs from SLE patients have been used. However during the period of this thesis no splenic tissue was available. Patients who had been
shown previously to have antibodies to hsp90 were selected. The fusion partner used was CB-F7 (a gift from Dr. Siegbert Jahn, Charité hospital, Berlin, Germany) found previously to be the most successful [Ehrenstein 1994].

Separation of lymphocytes from peripheral blood

Peripheral blood (50mls) was collected into heparinised tubes (10U/ml sodium heparin, Monoparin CP pharmaceuticals Wrexham UK). The blood was diluted with sterile PBS 1:1 and layered on the surface of Ficoll-Hypaque (Nycomed Oslo Norway) in 50ml Falcon tubes and centrifuged at 400g for 20 min., the ratio of diluted blood to Ficoll-Hypaque was 2:1. Lymphocytes were collected from the interface diluted with RPMI and washed with RPMI three times.

The cells were then resuspended in 1ml of RPMI and viable cells counted using a haemocytometer after staining with a 1:1 mixture of ethidium bromide and acridine orange. The cells were then placed in a modified counting chamber (Gallenkamp, Loughborough, UK.) and allowed to settle prior to counting.

No of Cells =Cells counted x 1/dilution x 10^4

Fusion with CB-F7

PBLs were fused directly with the mouse human heteromyeloma cell line CB-F7, a HAT sensitive ouabain resistant non-secreting cell line by PEG 1500 (Boehringer Mannheim Germany). CB-F7 cells were grown to log phase in serum (Appendix I). Prior to fusion CB-F7 cells were tested for HAT sensitivity by culturing for 24h in HAT medium and staining with ethidium bromide and acridine orange.
PBLs and CB-F7 cells were washed simultaneously in RPMI three times and cell numbers adjusted to give a ratio of CB-F7:PBL of 1:1. Mixed cells were centrifuged (200g for 10 minutes) in 50mls RPMI. Supernatant was pipetted off and the cells gently resuspended by tapping the bottom of the tube. 1ml of prewarmed 50% PEG in RPMI was added dropwise to every 5x10^6 cells approximately over the course of 1 minute. The tube was then gently rotated for 90 seconds and prewarmed RPMI was added very slowly over 8 minutes, to make a total volume of 50ml. The cells were then centrifuged again (200g for 10 minutes), resuspended in post fusion medium (Appendix I), and placed in 96 well micotitre plates, at a cell concentration of 105 cells/well, 100μl/well. After 24h, 100μl/well of 2x HAT medium was added to the wells. Growth was observed 7-10 days later. When the medium started to change colour it was replaced with fresh HAT medium.

Wells containing colonies covering a quarter of the base of the plate were tested for antibody secretion. Cells secreting an antibody of interest were transferred to 24 well plates (Nunc) and the cells allowed to expand. After further testing of supernatents the cells were then subcloned. At this point cells were transferred to HT medium and eventually to GM medium after a further three weeks. Cells secreting antibodies of interest were then subcloned twice more. The supernatents were screened for total IgG and IgM immunoglobulin levels and antibodies to the heat shock proteins and antibodies to DNA

Subcloning

This was achieved using limiting dilution, with the addition of mouse peritoneal macrophages as feeders. These cells were obtained from female BALB/c mice (Tuck, Battlebridge Essex). The cells were centrifuged (120g
for 10 minutes) at 4°C to prevent cells sticking to the plastic. The cells were washed twice, counted and resuspended into GM medium. The cells were plated out at $5 \times 10^3$ cells/well in 96 well plates. These were incubated for 48h to observe for possible infection.

Subcloning prevents overgrowth by faster non-secreting cell lines. If the cells are seeded at a low enough density the fraction of wells with growth should follow the Poisson distribution [Lefkovits and Waldman 1979]:

$$f(0) = e^{-\lambda}$$

where $f(0)$= fraction of cells with no growth

$\lambda$=average numbers of clones per well

Hybridoma cells from selected wells were counted and then diluted in medium to a density of 5 cells/ml, 10 cells/ml and 50 cells/ml. 200µl aliquots were placed in the central 60 wells of 96 well tissue culture plates. All wells with growing cells were checked for antibody secretion and specificity. Subcloning continued until all wells in which there were growing cells secreted the same antibody.

2.7. Detection of human Immunoglobulin

Goat anti-human IgG F(ab')$_2$ antibodies or goat anti-human IgM antibodies (Sigma) was coated onto ELISA plates, in BIC buffer at 1µg/ml overnight at 4°C. The plates were washed (BIC buffer) and blocked with 2% BSA (Sigma) for 1 hour at 37°C. After washing with PBS-T the supernatants were incubated in the plate for 1 h at 37°C. The conjugates and substrates were added and plates read as described previously.

ELISA to detect anti-DNA antibodies
ELISA plates were coated with poly-l-lysine hydrobromide (Sigma) at a concentration of 50μg/ml in distilled water for 1 h at 37°C. Plates were washed (PBS) and ssDNA, dsDNA and water were each coated in a third of the wells on each the plate. ssDNA and dsDNA (for preparation see appendix), were coated overnight at 4°C at concentrations of 5μg/ml and 10μg/ml respectively. Plates were washed (PBS) and neutralised with poly-l-glutamate (Sigma) for 1 hour at 37°C. After washing with PBS plates were blocked with 2% casein in PBS. Supernatants were screened at a 1 in 2 dilution. Appropriate positive and negative controls were included on each plate. Bound anti-DNA antibodies were detected using goat anti-human IgG and IgM alkaline phosphatase, incubated for 1 h at 37°C. The colour was developed using p-nitrophenyl phosphate tablets (5mg, Sigma) as substrate. Plates were then read as previously described. Any supernatant that appeared to be positive was always rescreened three days later before subcloning.
3.1 Validation of an assay to detect the presence of antibodies to hsp90.

Prior to designing an assay system to detect and quantify antibodies to human hsp90 it was essential to show that the commercially obtained hsp90 did not have any contaminants which might be recognised by other antibodies in the sera from patients. This was achieved by running the protein using PAGE, staining using Coomassie blue and destaining as described in the methods section. Initially, batches of hsp90 were shown to contain several bands or be partially degraded. However, eventually suitable batches of hsp90 became available which showed only one discrete band on a gel. Figs 3.1 and 3.2 demonstrate clearly the difference between degraded hsp90 (fig 3.1) and a discrete band of hsp90 (fig 3.2). Once this had been achieved Western blotting was used to ensure that the monoclonal antibody AC88 could react with this protein, confirming that it is indeed hsp90 (fig 3.3).

A suitable ELISA was then developed as described in chapter 2. The mAb AC88 did not bind the native hsp90 as well as AC16 in preliminary experiments. AC16 was shown to bind to native hsp90 in a dose dependent fashion (fig 3.4). Inhibition ELISAs demonstrated that binding of the mAb was inhibited by pre-incubation with hsp90 by up to 60%. A selection of sera
Fig 3.1. Coomassie gel of purified heat shock protein 90. Several bands are clearly visible. Therefore this batch of protein could not be used.

Lane A: Molecular weight markers
Lane B: HSP90
Lane C: HSP27
Fig 3.2. Discrete band of purified hsp90 on a Coomassie gel

Lane A: hsp90
Lane B: Molecular weight markers

Fig 3.3 Western blot showing binding of antibody AC88 to hsp90.
Fig 3.4 Dose dependent binding of AC16 to human hsp90 by ELISA. The experiment was repeated three times.

Fig 3.5 Inhibition ELISAs for hsp90. Each experiment was repeated three times.

1a  Positive control serum from SLE patient
1b  Positive control serum pre-incubated with hsp90
2a  AC16 antibody (monoclonal antibody)
2b  AC16 antibody pre-incubated with hsp90
from patients and healthy controls were then taken to optimise the ELISA conditions. Several of these sera were then used in an inhibition ELISA. These inhibition studies showed that sera binding to antigen on the plate could be reduced up to 60% by prior incubation with hsp90 (fig 3.5). From these preliminary results, sera were selected to act as the positive and negative controls for IgG and IgM antibodies to hsp90. These sera were then dispensed into smaller volumes, to avoid multiple freeze-thawing and frozen at -70°C until required. Each plate had a positive control of the correct isotype (IgG or IgM) with 2 sera that were 80% and 20% of the positive control as well as a negative control serum and a blank.

3.2 Antibodies to hsp90 in SLE, healthy controls and patients with other autoimmune diseases

Sera from 28 healthy sex-matched controls (27F,1M) were used to form the group of healthy controls and sera from 44 SLE patients were used to detect the presence and levels of IgG and IgM antibodies to hsp90. The levels of anti-hsp90 antibody were considered to be positive if greater than the mean +2SD of the healthy controls. These initial results demonstrated both the presence of these antibodies in SLE patients at a higher frequency than the healthy controls and showed that the mean levels were also higher (figs 3.6, 3.7). Thus these preliminary findings indicated that these antibodies were present in some SLE patients. These data were then extended to increase the numbers of SLE patients and to investigate the presence of these antibodies in patients with other autoimmune diseases and thus elucidate whether these results were typical in an autoimmune response or restricted to patients with SLE.
Fig 3.6 IgG antibodies to hsp90 measured by ELISA, expressed as a percentage of a positive control, in the sera of SLE patients and healthy controls. (mean values indicated by line)

Fig 3.7 IgM antibodies to hsp90 measured by ELISA, expressed as a percentage of a positive control, in the sera of SLE patients and healthy controls. (with mean values shown by line)
Figs 3.8 and 3.9 indicate that whilst these antibodies are not restricted to SLE patients they occur much less often in other autoimmune diseases and at lower levels. Thus, raised IgG autoantibodies to hsp90 were detected in 19 (26%) of the 72 SLE patients. IgM autoantibodies were raised in 25 (34%) of SLE patients. These figures are similar to those in the myositis group in which four patients (22%) had raised IgG antibody levels and six (33%) raised IgM levels. However, the actual values in the SLE group tended to be higher. Only two out of 17 (12%) of the SS patients, one out of 28 (4%) of the healthy controls and none of the RA, SC or OA patients had raised IgG anti-hsp90 antibody levels. In the IgM isotype assays only two out of 15 (13%) of the SC, 7% of the RA, one out of 28 (4%) of the healthy controls and none of the SS or OA patients had elevated levels of this antibody. The mean levels of antibodies in each of these patient groups are summarised in table 3.1. The mean levels of elevated IgG or IgM anti-hsp90 antibodies are higher in the control group than in patients with RA or scleroderma.

There was no correlation between IgG and IgM anti-hsp90 antibody levels in all patients with SLE (fig 3.10). In addition, antibody levels were examined for correlation when each antibody was considered to be positive i.e. greater than mean plus 2SD of the normal healthy controls since there were a significant number of patients who did not have detectable levels of antibodies. No correlation was found in this subset of patients where each serum sample had elevated levels of both antibodies. (fig 3.11). Rank correlations between IgG and IgM antibodies to hsp90 were also examined in the other autoimmune diseases studied but none were found.
Fig 3.8 IgG antibodies to hsp90 in patients with SLE, scleroderma (SC), Sjögren's syndrome (SS), myositis (MY), RA, OA, and healthy controls, (line is drawn at mean + 2SD of the healthy controls).
Fig 3.9 IgM antibodies to hsp90 in patients with SLE, scleroderma (SC), Sjögren's syndrome (SS), myositis (MY), RA, OA, and healthy controls, (line is drawn at mean + 2SD of the healthy controls).
Table 3.1. Mean IgG and IgM anti-hsp90 antibody levels in sera from patients with autoimmune rheumatic diseases.

<table>
<thead>
<tr>
<th>Patients</th>
<th>IgG Abs (SD)</th>
<th>IgM Abs (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls</td>
<td>0.3 (1.62)</td>
<td>3.5 (7.97)</td>
</tr>
<tr>
<td>SLE</td>
<td>4.73 (14.4)</td>
<td>17.4 (23.9)</td>
</tr>
<tr>
<td>SS</td>
<td>0.79 (2.03)</td>
<td>1.9 (4.13)</td>
</tr>
<tr>
<td>Myositis</td>
<td>3.15 (7.71)</td>
<td>19.4 (27)</td>
</tr>
<tr>
<td>RA</td>
<td>0.05 (0.19)</td>
<td>3.2 (6.94)</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>0.21 (0.59)</td>
<td>3.01 (7.95)</td>
</tr>
</tbody>
</table>

Value of each serum sample obtained as a percentage of the positive control
SD standard deviation
Fig 3.10 IgG anti-hsp90 antibodies versus IgM anti-hsp90 antibodies in 72 patients with SLE.

Fig 3.11 IgG anti-hsp90 antibodies versus IgM anti-hsp90 antibodies in patients with SLE with raised levels of antibodies of both isotypes.
Anti-hsp90 antibodies in relation to disease parameters in patients with SLE

Having established that there is a subset of patients who have elevated levels of antibodies to hsp90, clinical parameters were investigated for correlations with the presence of these antibodies. Chi square tests were utilised to examine whether those SLE patients who were positive for anti-hsp90 antibodies also had on the same occasion elevated levels of anti-dsDNA antibodies. This same approach was also undertaken to examine relationships between anti-hsp90 antibodies and ESR. No correlations were found with these parameters (table 3.2).

Using the BILAG system (as described in the Methods section), anti-hsp90 antibody levels were compared with overall disease activity in patients with SLE (table 3.3). Global activity was not found to correlate with anti-hsp90 antibody level. In contrast renal activity and serum C3 complement levels were found to correlate with the presence of anti-hsp90 antibodies (p<0.05). However, there was no correlation between anti-hsp90 antibodies with anti-DNA antibodies or ESR levels. There was also no correlation between the presence of rheumatoid factor and anti-hsp90 antibodies combined or of either isotype, nor was there any correlation with total immunoglobulin and antibodies to hsp90.

The clinical records of patients with SLE were examined for evidence of infection within the three months prior to serum collection. Only nine patients had an infection reported (table 3.4). Serum from only one of the patients diagnosed with an infection was negative for both isotypes of anti-hsp90 antibody, although IgG anti-hsp90 antibodies were elevated in 16 patients with SLE with no evidence of infection; and IgM antibodies elevated in 20 patients. Three healthy controls were also found to be positive for anti-hsp90
Table 3.2. Association between the presence of anti-hsp90 antibodies and clinical parameters in patients with SLE.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>hsp90 +ve</th>
<th>hsp90 -ve</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR+</td>
<td>24</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>ESR-</td>
<td>8</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>DNA+</td>
<td>19</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>DNA-</td>
<td>14</td>
<td>14</td>
<td>N/S</td>
</tr>
<tr>
<td>C3+</td>
<td>10</td>
<td>20</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>C3-</td>
<td>15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Cardio+</td>
<td>17</td>
<td>5</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Cardio-</td>
<td>13</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>RF+</td>
<td>7</td>
<td>7</td>
<td>N/S</td>
</tr>
<tr>
<td>RF-</td>
<td>24</td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

DNA Anti-dsDNA antibody level: + greater than 100, - less than 100 lu/l
C3 Complement level: + greater than 0.75, - less than 0.75g/l
ESR Erythrocyte sedimentation rate: + greater than 25, - less than 25mm in first hour. N/S not significant.
Cardio Cardiolipin antibodies. RF Rheumatoid Factor
Table 3.3 Association between the presence of anti-hsp90 antibodies and disease activity in patients with SLE.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Anti-hsp90 +ve</th>
<th>Anti-hsp90 -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>BILAG&gt;6</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>BILAG&lt;6</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>CNS+</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>CNS-</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>HAEM.+</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>HAEM.-</td>
<td>28</td>
<td>20</td>
</tr>
<tr>
<td>REN.+</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>REN.-</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>CAR/RES+</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>CAR/RES-</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>GEN.+</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>GEN.-</td>
<td>27</td>
<td>21</td>
</tr>
<tr>
<td>MUS/SKEL.+</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>MUS/SKEL.-</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>MUCCO.+</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>VASC.+</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>VASC.-</td>
<td>26</td>
<td>28</td>
</tr>
</tbody>
</table>

+ active disease BILAG score A, B
- inactive disease BILAG:CDE
Haem. haematological disease
Ren. renal disease
Car/Res cardio-respiratory disease
Neur. neurological disease
Musc/Skel muscular/skeletal disease
Glo. Global
Muco mucocutaneous disease
Vasc Vascular
CNS central nervous system
N/S not significant
Table 3.4 Analysis of hsp90 antibodies in patients with infection within two months of serum sample being taken

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgG</th>
<th>IgM+ve</th>
<th>Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>-</td>
<td>-</td>
<td>chronic scalp infection</td>
</tr>
<tr>
<td>P2</td>
<td>+</td>
<td>+</td>
<td>infected vascular ulcers</td>
</tr>
<tr>
<td>P3</td>
<td>-</td>
<td>+</td>
<td>chest infection</td>
</tr>
<tr>
<td>P4</td>
<td>+</td>
<td>-</td>
<td>chronic scalp infection</td>
</tr>
<tr>
<td>P5</td>
<td>-</td>
<td>+</td>
<td>persistent infection</td>
</tr>
<tr>
<td>P7</td>
<td>-</td>
<td>+</td>
<td>persistent kidney infection</td>
</tr>
<tr>
<td>P8</td>
<td>+</td>
<td>-</td>
<td>recurrent chest infection</td>
</tr>
<tr>
<td>P9</td>
<td>-</td>
<td>+</td>
<td>recurrent chest infection</td>
</tr>
</tbody>
</table>
antibodies, two of whom had also had a chest infection, within the month preceding serum collection.

When the levels of hsp90 protein were compared with the HLA haplotype in our SLE patients it was found that hsp90 was significantly elevated in patients who were A1/B8/DR3-negative compared to those who had this haplotype (p<0.05) [Dhillon et al. 1992]. The haplotypes of patients who had elevated levels of autoantibodies to hsp90 were therefore examined (table 3.5). The majority of patients who had raised autoantibody levels were also A1/B8/DR3 negative. The HLA status of the healthy controls was unavailable. This haplotype is commonly found in Caucasian patients with SLE but not in non-Caucasian patients with SLE. As all of the controls were Caucasian, a group of 15 non-Caucasian healthy control sera were tested. Anti-hsp90 antibody levels were measured in these healthy controls (figs 3.12, 3.13). It is clear that the non-Caucasian group have higher levels of antibodies to hsp90 as well as a greater frequency with elevated anti-hsp90 antibodies, particularly of the IgG isotype compared to the Caucasian healthy controls (table 3.6). However, the mean antibody level of these controls is lower than the SLE patients.

It was then attempted to correlate elevated hsp90 protein levels with the presence of anti-hsp90 antibodies. Unfortunately, the majority of serum samples taken on the same date as the protein levels were measured were unavailable. Those serum samples for which there was a protein level measured on the same date (n=41) were examined for correlations (using chi square) and also whether levels of protein correlated with the actual levels of anti-hsp90 antibody (using Spearman rank correlations).
Table 3.5 Immunogenetic analysis of anti-hsp90 antibodies in patients with SLE

<table>
<thead>
<tr>
<th>HLA type</th>
<th>Anti-hsp90 +ve</th>
<th>Anti-hsp90 -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1+</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>A1-</td>
<td>21</td>
<td>12</td>
</tr>
<tr>
<td>B8+</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>B8-</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>DR3+</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>DR3-</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>A1/B8+</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>Others</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>A1/DR3+</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Others</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>B8/DR3+</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Others</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>A1/B8/DR3+</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Others</td>
<td>26</td>
<td>12</td>
</tr>
</tbody>
</table>

N/S not significant
Fig 3.12 IgG antibodies to hsp90 measured by ELISA, expressed as a percentage of a positive control, in the sera of Caucasian, Non-Caucasian healthy controls and SLE patients (mean values indicated by line).

Fig 3.13 IgM antibodies to hsp90 measured by ELISA, expressed as a percentage of a positive control, in the sera of Caucasian, Non-Caucasian healthy controls and SLE patients (mean values indicated by line).
Table 3.6 Mean anti-hsp90 antibody levels to hsp90 in serum from Caucasian and non-Caucasian healthy individuals compared to patients with SLE.

<table>
<thead>
<tr>
<th></th>
<th>IgG Abs (SD)</th>
<th>IgM Abs(SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>4.73 (14.4)</td>
<td>17.4(23.9)</td>
</tr>
<tr>
<td>Caucasians</td>
<td>0.3 (1.62)</td>
<td>3.5(7.97)</td>
</tr>
<tr>
<td>Non-Caucasians</td>
<td>3.5 (5.8)</td>
<td>9.4(9.96)</td>
</tr>
</tbody>
</table>

SD Standard deviation
These results are summarised in table 3.7. The table shows that amongst patients whose PBMCs had elevated levels of hsp90 twice as many had elevated levels of antibodies to hsp90. However, the differences were not statistically significant probably because of the small sample size.

Anti-hsp90 antibody levels, from patients with SLE, were plotted against previously measured hsp90 protein level (figs 3.14, 3.15). There was no correlation in the levels of either isotype of antibody and protein level. One reason for this might be that very few of the paired samples (n=5) available had increased expression of hsp90 protein. However, in those serum samples that did have increased levels of IgM antibodies to hsp90 there appeared to be a tentative relationship with increased levels of hsp90 protein, although it is not statistically significant (fig 3.16).

Since hsp90 is associated with the receptor for glucocorticoid hormones and is thought to play a role in the cellular response to corticosteroids, the patients’ treatment at the time of autoantibody measurement was noted to determine whether steroids had any effect on the level of autoantibodies. The SLE patients were categorised according to their steroid treatment: no steroids; prednisolone <5mg; prednisolone >5mg alone, or prednisolone >5mg and other immunosuppressives. Each group was compared with respect to the presence or absence of elevated anti-hsp90 antibodies (table 3.8). No correlation of elevated antibody levels with steroid therapy or the level of steroid administered was observed in the SLE patients studied.

Samples from human kidney eluates and eluates from MRL/lpr-lpr mice became available (courtesy of Professor Michael Madaio) and were tested
Table 3.7 Association between the presence of anti-hsp90 antibodies with surface expression of hsp90 and raised hsp90 protein levels in the peripheral blood mononuclear cells form patients with SLE.

<table>
<thead>
<tr>
<th>Hsp90 protein</th>
<th>Anti-hsp90 antibodies</th>
<th>hsp90 +ve</th>
<th>hsp90 -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMCs+</td>
<td>9</td>
<td>4</td>
<td>N/S</td>
</tr>
<tr>
<td>PBMCs-</td>
<td>14</td>
<td>13</td>
<td>N/S</td>
</tr>
<tr>
<td>Surf.+</td>
<td>9</td>
<td>8</td>
<td>N/S</td>
</tr>
<tr>
<td>Surf.-</td>
<td>11</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Protein: hsp90 protein elevated in the PBMCs at date of, or within four weeks of the serum sample taken. Protein quantified by Norton or Dhillon between 1989 and 1991 [Dhillon VB. 1993]
Fig 3.14 IgG antibodies to hsp90 versus hsp90 protein levels in paired samples from patients with SLE.

Fig 3.15 IgM antibodies to hsp90 versus hsp90 protein levels in paired samples from patients with SLE.
Fig 3.16 Hsp90 protein level versus IgM anti-hsp90 antibodies in paired samples from patients with SLE and elevated levels of IgM antibodies to hsp90.
Table 3.8 Analysis of steroid treatment in relation to anti-hsp90 antibody level in patients with SLE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anti-hsp90 +ve</th>
<th>Anti-hsp90 -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>No steroids</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Prednisolone &lt; 5mg</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Prednisolone &gt; 5mg and other immunosuppressives</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>
for the presence of anti-hsp90 antibodies. In a very limited study examining
the presence of these antibodies in human kidney eluates (n=3) very low
levels of anti-hsp90 antibody was detected in one of these samples.
Unfortunately there was insufficient sample for duplicate testing so no firm
conclusions can be drawn. With the MRL/lpr--lpr mice none of these eluates
appeared to be positive for hsp90 antibody reactivity (n=6).

Sera were available from 25 first degree relatives of several patients with
SLE. Four of these relatives were positive for IgG anti-hsp90 antibodies and
five of these relatives were positive for IgM anti-hsp90 antibodies. First
degree relatives of these patients appeared to have higher levels of these
antibodies than relatives from patients who had no increased elevation of
these antibodies although this relationship failed to reach statistical
significance probably due to small sample size. The increased levels of anti-
hsp90 antibodies in these relatives was most notable in the non-Caucasian
group.

Serial studies were then undertaken in several patients. Figs 3.17 and 3.18
demonstrate that antibody levels fluctuate over time and can be one isotype
(as in patient 1, fig 3.17) or different isotypes (as in patient 2, fig 3.18). Fig
3.18 shows all three isotypes to hsp90 antibody (IgG, IgA and IgM)
parallelling each other. Therefore a patient can be positive at one time but
negative several months later. These results indicate that levels of antibody
are variable over a time and a patient can develop one or both isotypes. Figs
3.19 (patient 3) and 3.20 (patient 2) show the levels of C3, ESR and anti-
DNA antibodies at these time points. Fig 3.21 (patient 4) shows an example
of a correlation between IgG antibody levels and ESR level in one particular
Fig 3.17 shows fluctuation of IgM anti-hsp90 antibodies in an SLE patient (P1) over time. This patient was never positive for IgG anti-hsp90 antibodies.

Fig 3.18 shows fluctuation of IgA, IgG and IgM anti-hsp90 antibodies in an SLE patient (P2) over time.
Fig 3.19 shows the variation of IgG anti-hsp90 antibody level with anti-dsDNA antibodies, ESR and C3 levels in one patient with SLE (P3). This patient had the highest levels of IgG anti-hsp90 antibodies, but was always negative for IgM anti-hsp90 antibodies. The Y axis is arbitrary and shows relative levels of each parameter.
Fig 3.20 shows the variation of IgG, IgA and IgM anti-hsp90 antibodies with anti-dsDNA antibody level and ESR level in one patient (P2) with SLE.
Fig 3.21 Correlation between IgG anti-hsp90 antibodies versus ESR level in one patient with SLE (P4). Similar relationships were found in four other patients with SLE.

Fig 3.22 shows the relationship between IgM anti-hsp90 antibodies and levels of hsp90 protein in one patient with SLE (P5). This patient was always negative for IgG anti-hsp90 antibodies.
patient. This correlation has been shown in several patients (n=4) over a similar time course. Only one set of paired sera (patient 5) showed a clear relationship between antibody level and actual hsp90 protein, and this was with IgM antibodies only (fig 3.22). In all these longitudinal studies the scales are completely arbitrary and are designed to show the relative values of each parameter and any possible relationship between these parameters.

3.3 Antibodies to hsp70

Having established that antibodies to hsp90 can be detected in SLE patients and that these antibodies are elevated in a proportion of these patients compared to healthy controls, it was important to consider whether these antibodies arise as part of a general anti-hsp response or whether they are a specific anti-hsp90 response. An ELISA was developed to investigate the presence of antibodies to mammalian hsp70 in SLE patients and healthy controls. The ELISA was established in the same way as that described for hsp90. A band at 70kDa is visible in the Coomassie gel in fig 3.23. Again this ELISA was established with the 17 controls (16F,1M) and a serum sample was considered to be positive for hsp70 antibodies if the value obtained was greater than that of 17 healthy controls plus two standard deviations. Inhibition ELISAs demonstrated that binding of the mAb was inhibited by pre-incubation with hsp70 by up to 50% (fig 3.24).

The results examining the presence of anti-hsp70 antibodies are shown in table 3.9 (figs 3.25 and 3.26). This data demonstrates that these antibodies do occur in the sera of SLE patients but at a much lower frequency than anti-hsp90 antibodies. Moreover, the overall mean levels of anti-hsp70
**Fig. 3.23** Heat shock protein 70 on a Coomassie gel showing a clear discrete band.

Lane A: Molecular weight markers
Lane B: HSP 90
Lane C: HSP 70
Lane D: HSP 27

**Fig. 3.24** Inhibition ELISAs for hsp70. Each experiment was repeated three times.

1a  Anti-hsp70 antibody (monoclonal antibody)
1b  Anti-hsp70 antibody pre-incubated with hsp70
2a  Serum from SLE patient
2b  Serum from SLE patient pre-incubated with hsp70
Table 3.9 Mean antibody levels to hsp70 in patients with SLE, OA, RA, and myositis and in healthy controls.

<table>
<thead>
<tr>
<th>Patients</th>
<th>IgG Abs (SD)</th>
<th>IgM Abs (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls</td>
<td>0.86 (1.16)</td>
<td>0.72 (1.31)</td>
</tr>
<tr>
<td>SLE</td>
<td>0.61 (1.46)</td>
<td>0.58 (1.17)</td>
</tr>
<tr>
<td>RA</td>
<td>0.9 (1.37)</td>
<td>0.79 (1.03)</td>
</tr>
<tr>
<td>OA</td>
<td>0.87 (2.3)</td>
<td>0.3 (0.79)</td>
</tr>
<tr>
<td>Myositis</td>
<td>0.63 (0.89)</td>
<td>0.65 (1.08)</td>
</tr>
</tbody>
</table>

SD Standard deviation
Fig 3.25 IgG antibodies to hsp70 measured by ELISA, expressed as a percentage of a positive control, in the sera of SLE patients and healthy controls. (with mean values shown by line)

Fig 3.26 IgM antibodies to hsp70 measured by ELISA, expressed as a percentage of a positive control, in the sera of SLE patients and healthy controls (with mean values shown by line).
antibodies are actually lower in SLE than healthy controls since the majority of values for antibodies to hsp70 in sera from SLE patients are zero.

Elevated IgG and IgM anti-hsp70 antibodies are present in 8.9% and 7.1% of SLE patients respectively. The correlation between IgG and IgM anti-hsp70 antibodies in SLE is shown in fig 3.27. No statistical association was found.

Sera from patients with RA, OA and myositis were examined for antibodies to hsp70. The frequency of raised anti-hsp70 antibodies in myositis patients was lower than the frequency of anti-hsp90 antibodies, although in RA patients the frequency of patients with raised anti-hsp70 antibodies was higher than the frequency of samples with elevated anti-hsp90 antibodies (figs 3.28, 3.29).

Graphs of IgG anti-hsp70 versus IgG anti-hsp90 and IgM anti-hsp70 versus IgM anti-hsp90 were plotted. No correlation was observed (fig 3.30, 3.31). No correlation between IgG hsp90 antibodies and IgM anti-hsp70 antibodies, or IgM anti-hsp90 antibodies and IgG anti-hsp70 antibodies was found (figs 3.32, 3.33).

3.4. Attempt to generate monoclonal anti-hsp90 antibodies from SLE patients

Having established that anti-hsp antibodies were detectable in the sera from patients with SLE, and attempt was made to generate monoclonal anti-hsp antibodies from those patients who expressed high levels of anti-hsp antibodies. It had been shown that patients who had active disease were
Fig 3.27 Graph showing no relationship between the levels of IgM anti-hsp70 antibodies and IgG anti-hsp70 antibodies in the sera from patients with SLE.
Fig 3.28 IgG antibodies to hsp70, in the sera of patients with SLE, RA, myositis (MY), OA and healthy controls. (means shown by line).

Fig 3.29 IgM antibodies to hsp70, in the sera of patients with SLE, RA, myositis (MY), OA and healthy controls. (means shown by line).
Fig 3.30 IgG anti-hsp70 antibodies versus IgG anti-hsp90 antibodies in paired samples from 56 patients with SLE.

Fig 3.31 IgM anti-hsp70 antibodies versus IgM anti-hsp90 antibodies in paired samples from 56 patients with SLE.
Fig 3.32 IgG antibodies to hsp90 versus IgM antibodies to hsp70 in paired samples from 56 patients with SLE.

Fig 3.33 IgM antibodies to hsp90 versus IgG antibodies to hsp70 in paired samples from 56 patients with SLE.
generally more likely to produce IgG secreting B cells (Ehrenstein et al. 1992). Thus, where possible, patients who had a global BILAG score greater than six were selected. In addition all patients whose peripheral blood lymphocytes were used had elevated anti-hsp antibodies on their most recent visit to the clinic. As shown in figs 3.17 and 3.18 anti-hsp90 antibodies fluctuate considerably over time. Therefore, wherever possible, patients hospitalised with active disease had their anti-hsp90 antibody level assessed 24hrs prior to fusion. If this proved impractical, a serum sample was taken at time of fusion and subsequently tested for anti-hsp90 antibodies.

In total 18 fusions were performed on 17 SLE patients and 1 laboratory colleague who had been shown previously to have elevated anti-hsp90 antibodies, over a period of seven months. Of these 18 fusions, 12 were successful in that they generated at least one clone that secreted immunoglobulin (G or M) (table 3.10). All clones were screened for total immunoglobulin, if positive they were then rescreened three days later, for each isotype (IgG and IgM). If there were any positives on repeat testing three days later clones were tested for anti-DNA antibodies (both ssDNA and dsDNA of either isotype), as well as anti-hsp antibodies. Since there is only about 150μl of supernatant available in each well for testing, and it was not practical to expand all secreting clones, a mixture of hsp90 were coated on to each plate. Any clone that appeared to be positive for the hsp mixture was then retested against the individual hsp90s (table 3.11) Initially the concentration of antigen used to establish the ELISA was taken, but it is likely that the initial concentrations selected were too low for the detection of an early clone secreting anti-hsp antibodies.
Table 3.10 Summary of patients with SLE selected for fusion.

<table>
<thead>
<tr>
<th>P.</th>
<th>No. clones</th>
<th>No. secreting clones</th>
<th>BILAG</th>
<th>Sera hsp90+ve on same date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>IgM</td>
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P=patient, C=control, N/A=not applicable
Table 3.11 Antibody secreting clones from fusions which resulted in clones

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<th>ssDNA G M</th>
<th>dsDNA G M</th>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

P=patient, C=control
Thus after initial screening, the fusion of one patient who had been hospitalised due to severe infection, resulted in either one clone producing antibodies that recognised all three hsp antibodies or a well containing several clones (P7). All three clones were of the IgM isotype. Fusions from two other patients also generated clones that produced anti-hsp70 and anti-hsp27 antibodies. The anti-hsp70 antibody was of the IgM isotype and the anti-hsp27 was of the IgG isotype. The lymphocytes from the laboratory colleague, who had high levels of IgG anti-hsp90 and detectable levels of anti-hsp70 antibody failed to generate any anti-hsp clones following fusion. Potential clones were then rescreened and subcloned. After two sets of subcloning the clone from P7 stopped secreting immunoglobulin. Vials that had previously been frozen were thawed, initially they thrived but again failed to maintain secretion of the antibody. The clone that appeared to secrete anti-hsp70 antibody also stopped secreting immunoglobulin after one round of subcloning, although it is not absolutely clear that the antibody produced by this clone was against hsp70 since repeated testing resulted in ambiguous results. An infection in the incubator resulted in the clone secreting anti-hsp27 becoming contaminated prior to subcloning for the first time. Any clone that was secreting antibody and tested negative for anti-hsp antibodies, was then tested for secretion of another antibody of interest in the laboratory, anti-cardiolipin antibody, by Dr. Sanj Menon. The first human IgG anti-cardiolipin antibody to be generated resulted from this fusion.
3.5 Discussion

3.51 Establishing a suitable method for detecting antibodies to hsp90

The initial aim of this work was to develop a simple, but accurate method for detecting antibodies to human hsp90. A suitable ELISA was established using native human hsp90 and demonstrated these antibodies in patients with SLE. The results described here demonstrate that 34% of SLE patients have raised IgM anti-hsp90 antibodies and 26% IgG antibodies. Thus approximately one-third of lupus patients had raised autoantibody levels to hsp90. Previous studies have reported antibodies to hsp90 in 47% patients with SLE and 33% of patients with myositis but not in sera from healthy controls, or patients with RA or scleroderma [Minota et al. 1988b]. These results were obtained by SDS PAGE followed by Western blotting with the source of hsp90 being denatured human T cell lysates. These autoantibodies were exclusively of the IgG isotype.

Subsequently however, these authors reported rather different results using a similar method of immunoblotting [Jarjour et al. 1991]. They described a much lower frequency of autoantibodies: one of 48 SLE patients had IgG anti-hsp90 antibodies and six anti-hsp73 antibodies; no RA patients had anti-hsp90 antibodies and only one RA patient had hsp73 antibody. In the patients with myositis, the only antibody detected was to hsp60 (GroEL). In the healthy controls 3% had anti-73 and anti-hsp60 antibodies, but anti-hsp90 antibodies of either isotype were not detected.

There are several differences in these experiments undertaken by the same
group. Firstly, assay was difficult to standardise, especially since the authors reported that sensitivity varied greatly with incubation of blots and therefore selected an arbitrary time point for incubation with sera assuming that a normal control serum would be unlikely to have anti-hsp antibodies and the authors fail to mention how many control sera they selected to establish these conditions. Secondly, the sera was treated differently in both sets of experiments; in the former they were heated for a considerable length of time which may affect the stability of the antibody.

There may be several reasons for the discrepancies seen in the results reported by Winfield and colleagues compared to those described here. Firstly, the antigen used in these experiments was used in its native form, whereas in the experiments by Winfield and colleagues denatured antigen was used. There may be some anti-hsp90 antibodies that are able to recognise exclusively the denatured or native form of this antigen. In addition, ELISAs are a far more sensitive technique of detecting the presence of antibodies than Western blotting. Interestingly when purified antigen was blotted and incubated with sera known to be positive for anti-hsp90 antibodies only a very faint band could be seen, implying that the epitopes of hsp90 detected by the sera might either be hidden or destroyed by denaturing the protein. There was insufficient sera available for multiple attempts at different concentrations for immunoblotting since the volume of sera required for Western blotting is far greater than that needed for an ELISA. One major advantage of using an ELISA rather than Western blotting for detection of these antibodies is that antibody levels can easily be quantified since values have been expressed relative to a positive control. No previous attempt has been made to actually semi-quantify serum levels of
This study shows that there is significant overexpression of antibodies to hsp90 in patients with SLE. Antibodies to hsp90 were detected in a significant numbers of patients with SLE; 26% had elevated IgG antibodies and 34% had elevated IgM antibodies, which is a significant number of patients in as heterogeneous a disease as SLE, and is similar to the number of patients with SLE who for example have anti-Ro, anti-RNP antibodies and anti-Sm antibodies, the last being one of the criteria for classifying the disease [Tan et al. 1982]. Antibodies to hsp90 were detected rarely in patients with other autoimmune rheumatic diseases studied, implying that these antibodies are not part of a general autoimmune response. In fact the levels of antibodies to hsp90 were considerably lower in patients with RA and scleroderma than in healthy controls. The results that antibodies to
hsp90 were detected at higher frequency in first degree relatives of patients with SLE compared to healthy controls, particularly in the relatives of non-Caucasians, suggests that there might well be a genetic and environmental predisposition to develop these antibodies. Similar results have been published with data comparing levels of anti-ssDNA antibodies, and anti-nuclear antibodies in relatives of patients with SLE compared to a control group [Miles and Isenberg 1993]; although only one study found significant numbers of relatives with anti-dsDNA antibodies using an ELISA technique whereas other assay systems used radioimmunoassay and *Crithidia luciliae*, much less sensitive techniques [Le Page et al. 1989].

In contrast to the levels of anti-hsp90 antibodies detected, the results presented here demonstrate that elevated anti-hsp70 antibodies were found in less than 10% of all SLE patients studied. These antibodies to hsp70 were actually detected by using a mixture of of both purified mammalian native hsp72 and hsp73. It would therefore be of interest to develop suitable methods of detecting antibodies to hsp72 and hsp73 in order to establish whether the antibodies are directed against the constitutive or inducible form of this antigen. A recent report has demonstrated a link between antibodies to the constitutive native 73kD hsp (but not the inducible 72kD isoform) in mixed connective tissue disease [Mairesse et al. 1994]. These antibodies were also detected at elevated levels in patients with scleroderma, RA but not in patients with SLE or myositis. A previous study of adult SLE patients, utilising hsp70 derived from a human fibrosarcoma cell line HT-1080, found IgG and IgM anti-hsp70 antibodies raised in 20-30% of both patients and controls [Kindas-Mügge et al. 1993].
In general, the studies describing the presence of antibodies to hsp70 and hsp90 in patients with autoimmune diseases are somewhat contradictory, not least because the antigen used often contains bacterial contaminants which could result in the detection of anti-bacterial antibodies rather than anti-hsp antibodies. The antigen used in these experiments is purified mammalian hsps rather than a recombinant protein and therefore less likely to contain bacterial hsp.

In contrast to the levels of anti-hsp90 antibodies detected elevated, anti-hsp70 antibodies were found in less than 10% of all SLE patients. No correlation was found between the levels of antibodies to hsp90 and hsp70 in individual samples. There was also no correlation between the presence of IgG antibodies and IgM antibodies at the same time in individual samples, although there are several examples of serial trends in some SLE patients, such as in fig 3.18 where the levels of IgG, IgM and IgA antibodies to hsp90 paralleled each other. There was no correlation between PBMC samples with elevated hsp90 protein and those patients having elevated antibodies to hsp90 on the same date, however, caution is required in interpreting these results as very few paired samples were available for testing (or within one month of sera being available). Interestingly, PBMCs from patients with myositis have been shown to overexpress hsp90 (p<0.01) [Dhillon 1993]. Sera from patients with myositis were also shown to have increased levels of IgG and IgM anti-hsp90 antibodies.

Elevation of transcription of the hsp70 gene did occur in some patients, but not in the same patients with elevated hsp90β transcription, and there was no correlation between hsp90β transcription and that of the the hsp70 gene,
implying that post-transcriptional mechanisms may be responsible for the elevation of hsp72 protein observed in SLE. There was also no increase in transcription of the ubiquitin gene which provides additional evidence for differential overexpression of hsps in SLE [Twomey et al. 1992].

Thus it appears that the production of antibodies to hsp90 is not simply part of a generalised stress response in autoimmune rheumatic diseases. Rather it is a specific response that parallels increased surface expression of hsp90 or a component of it, increased hsp90 in the PBMCs of patients with SLE and increased transcription of the hsp90β gene in these patients.
This thesis has demonstrated that antibodies to hsp90 are significantly elevated in patients with SLE. However, there was no correlation with global disease activity using the BILAG scoring system. In contrast, patients with elevated hsp90 protein levels were shown to have overall active disease [Norton et al. 1989; Dhillon et al. 1992].

The FACS analysis of shsp90 demonstrated significantly higher lymphocyte shsp90 in patients with active disease (BILAG global score >6, p<0.009) [Erkeller-Yüksel et al. 1992]. However, the only significant correlation between shsp90 and individual BILAG categories was with active musculo-skeletal disease (p<0.002), although there was no elevated hsp90 in this subgroup of patients. Thus, the patients with active musculo-skeletal SLE in whom shsp90 was elevated were unlikely to have had elevated total hsp90 [Dhillon 1993].

There was no correlation between those patients with SLE that were positive for anti-hsp90 antibodies and those patients who had elevated levels of anti-dsDNA antibodies. There was also no correlation between the actual titres of these antibodies in the same patient. In fact in some of the patients with SLE where serial studies were carried out, there appeared to be a trend that as the anti-hsp90 antibody titre increased, the levels of anti-dsDNA antibodies declined, which implies that anti-hsp90 antibodies are not simply a reflection of increased immunoglobulin. Thus the presence of these antibodies does not appear to follow a similar pattern of global activity. However, a correlation between elevated antibody level (IgG and IgM combined) and a low C3 level (often an indicator of active renal disease) was observed (p<0.05).
Analysis of individual organ or system involvement in these SLE patients using the BILAG categories as described in chapter 1 section was undertaken comparing the presence of absence of autoantibodies to hsp90. Only one correlation was found, with IgM autoantibodies being elevated in patients with active renal disease (p<0.05). However in a very limited study examining the presence of these antibodies in human kidney eluates (n=3) very low levels of anti-hsp90 antibody was detected in only one of these samples. Unfortunately there was insufficient sample for duplicate testing so no firm conclusions can be drawn. With the MRL/lpr--lpr mice none of these eluates appeared to be positive sample although there was insufficient sample for testing in duplicate. It would be interesting to obtain kidney eluate from more patients, particularly since there appears to be an association between active renal disease and anti-hsp90 antibodies, as well as a decrease in C3 levels in patients positive for these antibodies, to elucidate whether there are antibodies to hsp90 at a site of pathogenesis. Immunohistochemical staining of hsp72 in kidney sections demonstrated no staining where the mechanism of injury was primarily immunological (lupus nephritis). These findings suggest that immunological damage may not be an important factor for the induction of the stress protein response [Dodd et al. 1993].

No correlation of elevated antibody levels with steroid therapy or the level of steroid administered was observed in the SLE patients studied. However, the majority of patients who had raised autoantibody levels were A1/B8/DR3 negative. This observation parallels the data on overexpression of the hsp90 protein itself which was also linked to patients with SLE who were HLA
A1/B8/DR3 negative. The importance of this observation is that the A1/B8/DR3 haplotype is the one most commonly found in Caucasian lupus patients, who constitute 60% of the patient group studied. This may be part of the explanation why a minority of the patients studied here appear to overexpress the antibody since the majority of our SLE patients possess this haplotype. Two-thirds of non-Caucasian patients with SLE were found to have elevated levels of anti-hsp90 antibodies, compared to the situation in Caucasian patients with SLE where a third of patients had elevated levels of anti-hsp90 antibodies. Interestingly, after observing that there appeared to be a bias in that patients with SLE were more likely to have elevated anti-hsp90 antibodies, if they were non-Caucasian sera from non-Caucasian healthy controls were taken and anti-hsp90 antibodies were detected. Table 3.6 shows that in this limited study, non-Caucasian healthy controls tended to have higher levels of this antibody, although the mean levels are well below that of the patients with SLE. Therefore the explanation for having elevated antibodies to hsp90 is unlikely to be simply having a particular haplotype. It is however possible that having this haplotype might make one genetically more susceptible to a particular type of infection, or to elevating hsp90 in response to an infection, which in turn might result in the production of antibodies to hsp90.

3.53 Possible Mechanisms resulting in the production of anti-hsp90 antibodies in patients with SLE

Antibodies to hsp90 might arise following the surface expression of the normally intracellular hsp90. Surface expression of hsp90 has been reported to occur on the lymphocytes and monocytes of patients with SLE, compared
to normal controls (p<0.006), using MAb AC88 and fluorescent activated cell sorting (FACS) analysis [Erkeller-Yüksel et al. 1992]. Monocytes from patients with SLE had a higher level of surface expression of hsp90 (shsp90) but this did not reach statistical significance. There appeared to be a heterogeneous pattern of shsp90 expression in different cell types occurring on monocytes, B cells, CD3+ T cells and CD4+ but not CD8+T cells. This parallels the finding that over expression of the hsp90 protein levels were differentially expressed in different cell types. Where surface expression of hsp90 was detected in lymphocytes, there was some correlation with hsp90 levels in the PBMCs.

There are several reasons why hsp90 or some component of it might be present at the cell surface; possibly as a result of chaperoning other proteins to the cell surface or as a result of its direct involvement in antigen presentation. It is also possible that hsp90 could be released from damaged cells and bind to viable cells. Therefore, due to either increased intracellular expression or an aberrant transport mechanism in SLE lymphocytes, hsp90 might become detectable on the cell surface where it could cause the activation of B or T cells.

Alternatively, hsp90 might play a more direct role in antigen presentation, which might explain the cell localisation of hsp90 or some component of it. It has been shown that peptides derived from both hsp90α and hsp90β are among several endogenous proteins whose peptides are bound to HLA-B27 in a lymphoblastoid cell line [Jardetzky et al. 1991]. Fibroblasts infected with herpes simplex virus accumulate high levels of hsp90 during lytic infection, some of which becomes located on the cell surface [LaThangue and
Latchman 1988]. In another study several tumour lines were found to express 2-10 times higher levels of hsp90 when compared to normal cells, and the hsp90 was localised to the cell surface [Ferrarini et al. 1991]. Interestingly, in immunoprecipitation experiments, this hsp90 co-precipitated with MHC 1 molecules, again suggesting a link with antigen presentation. Thus, it appears that in a number of different situations of hsp90 overexpression the excess protein becomes localised to the cell surface, either by an active mechanism or simply reflecting increased whole cell levels, where it may become involved, inadvertently, in an autoimmune process.

The question then arises of how this overexpression of hsp90 might occur. It has been demonstrated that hsp90 gene expression in human T cells is driven by an IL-2/IL-2 receptor dependent pathway. Mouzakai et al. [1992], demonstrated that the IL-2 gene ‘transcriptional silencer’ is inactive in lupus T cells, which might result in increased expression of hsp90. Interestingly, although hsp90α and hsp90β genes are both up-regulated by heat shock and mitogen stimulation, hsp90α is induced to a greater degree by heat shock, and hsp90β is more inducible by mitogen stimulation [Hansen et al. 1991]. Thus is is possible that the upregulation of hsp90β observed in patients with SLE could be mediated via cytokine stimulation, or it may be due to a primary genetic aberration, rather than a non-specific response caused by the stress of the disease.

Studies performed investigating hsp expression in MRL/lpr-lpr mice have also shown that hsp90 and hsp72, but not hsp73 or hsp60 are elevated in the spleen of these mice compared to MRL/++ mice or BALB/c mice. The
elevation in hsp90 levels in the MRL/lpr/lpr mice occurred prior to the onset of overt clinical or serological disease (as judged by anti-DNA antibody titres) at two months of age [Faulds et al. 1993]. There appeared to be different expression patterns for hsp90 and hsp72. Thus, the hsp90 elevation in spleen was shown to occur in a tissue specific manner, in contrast to the elevation of hsp72 which occurred in several different tissues including heart, brain, kidney and liver. Parallel experiments were carried out to detect the presence of anti-hsp90 antibodies in MRL/lpr/lpr mice [Faulds et al. 1994]. At twelve weeks of age at least 60% of these mice had elevated levels of these antibodies. Thus the same situation of over-expression of hsp90 protein is paralleled by the production of antibodies to hsp90. These results suggest that there might be a genetic predisposition in these mice to overexpress hsp90 resulting in the production of antibodies to it.

It is also possible that these antibodies are not in fact directed against human hsp90 but recognise some conserved epitope of hsp90. One possibility could be that these antibodies are actually directed against bacterial hsp90 but since hsp90 is so highly conserved between species, the antibodies recognise the human form of this protein. If this were the case, it might explain why eight out of the nine patients with SLE who had an infection within three months of the serum sample being taken were actually positive for at least one isotype of this antibody. Although, there were 16 and 20 patients with SLE that had elevated levels of IgG and IgM anti-hsp90 antibodies respectively. It would be interesting to obtain bacterial hsp90 and use it in inhibition assays to see whether it can compete out human hsp90. Interestingly, two out of the three laboratory controls who had elevated levels of hsp90 antibodies were both recovering from chest infections. Even if the
antibodies were initially directed against bacterial hsp90 there is must be some cross reactivity with the human homologue which could result in an autoimmune response.
CHAPTER 4

ANTIBODIES TO HSP90 AND HSP70 IN JUVENILE PATIENTS WITH AUTOIMMUNE RHEUMATIC DISEASES.

4.1 Antibodies to hsp90 in juvenile patients with autoimmune rheumatic diseases.

Sera from juvenile patients with SLE were screened for antibodies to hsp90 and hsp70. Serum controls were available from patients with juvenile chronic arthritis (JCA), dermatomyositis and non-autoimmune age-matched healthy controls. Table 4.1 shows the frequency of these antibodies to hsp90 and hsp70 in these samples. Tables 4.2 and 4.3 show the clinical features of these patients who are antibody positive and negative. Synovial fluid from patients with JCA was also available, and tested for antibodies to hsp90 and hsp70, although not from the same patients from whom sera was obtained.

The mean antibody values of the positive sera were very similar in the various disease groups, with the one notable exception being in the synovial fluid where the mean level of IgG anti-hsp70 antibodies was much higher. Antibodies reactive with hsp90 and/or 70 were found in 35% of the children with SLE; two patients had antibodies reactive with both hsp70 and hsp90. Nine children had elevated levels of antibodies to hsp90; eight (23.5% of the total SLE group) had IgG antibodies, whilst one (3% of the SLE group) had elevated levels of IgM anti-hsp90 antibodies. Fewer patients demonstrated reactivity with hsp70; three (8.8%) had IgG anti-hsp70 antibodies and two (5.8%) had IgM anti-hsp70 reactivity. Interestingly, IgM hsp90 antibodies were detected in these patients but at very low titres. These results for anti-
Table 4.1 Frequency of reactivity to hsp90 and 70 among children with SLE, JCA, DMS and controls

<table>
<thead>
<tr>
<th></th>
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<th>JCA</th>
<th>DMS</th>
<th>CONTROL</th>
<th>SYN FL.</th>
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<td>No.</td>
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<td>55</td>
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Anti-hsp90 antibodies

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<th>IgM</th>
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</tr>
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</tr>
<tr>
<td>DMS</td>
<td>3 (25)</td>
<td>0</td>
</tr>
<tr>
<td>CONTROL</td>
<td>2 (10)</td>
<td>1 (5.2)</td>
</tr>
<tr>
<td>SYN FL.</td>
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Anti-hsp70 antibodies

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<th>IgM</th>
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<tbody>
<tr>
<td>SLE</td>
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<td>2 (5.9)</td>
</tr>
<tr>
<td>JCA</td>
<td>3 (5.4)</td>
<td>3 (5.4)</td>
</tr>
<tr>
<td>DMS</td>
<td>4 (30)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>CONTROL</td>
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<td>1 (5.2)</td>
</tr>
<tr>
<td>SYN FL.</td>
<td>6 (50)</td>
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</table>

Results recorded as number of positive patients (% positive). i.e greater than the mean +2SD of the healthy controls
Syn=Synovial fluid
Table 4.2 Clinical characteristics of childhood-onset SLE patients with hsp70 and hsp90 antibodies

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<tr>
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<td>3.7</td>
<td>2.8</td>
</tr>
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</tr>
<tr>
<td>Rash</td>
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<td>Heart/Lung</td>
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</tr>
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</tr>
<tr>
<td>Renal</td>
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<td>10</td>
</tr>
<tr>
<td>Haematology (major)^</td>
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<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Fever</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Infection*</td>
<td>25</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Abnormal Serology\°</td>
<td>50</td>
<td>30</td>
<td>45</td>
</tr>
</tbody>
</table>

IgG and IgM isotypes are combined in the HSP70 and HSP90 columns.
# = 2/4 patients with hsp70 antibodies also were + for hsp 90.
* = bacterial infection within 3 months of serum sample.
° = abnormal serology refers to low serum complement and elevated anti DNA at the time of serum sample.
\^ = major haemolytic anaemia or profound thrombocytopenia [<50 x 10^9/L]
hsp70 antibodies in the juvenile patients are very similar to those in the adult patients.

There was no significant difference in age, or disease duration between these patients with SLE, antibodies to hsp90 or 70 and those without hsp reactivity. Patients with anti-hsp antibodies showed an increase in renal disease (although it was not statistically significant), whereas there were no differences in joint inflammation, rash, cardiopulmonary disease or central nervous system disease. There were no differences in abnormal serological values at the time of sampling between the positive and negative groups (table 4.2). The patients who were positive for hsp antibodies (anti-hsp70 and anti-hsp90 antibodies combined) were more likely to have had an infection within three months of serum sampling than those who were antibody negative; the infections seen were sinusitis, urinary tract infection and *Salmonella*.

4.2 Antibodies to hsp90 and 70 in patients with juvenile chronic arthritis (JCA)

Antibodies to hsp 70 and 90 were found in 23.6% (13 out of 55) of these patients. The numbers of children with IgG or IgM anti-hsp70 or 90 antibodies are shown in table 4.1. No child with JCA had both anti-hsp70 and 90 antibodies. Of the hsp reactivities, anti-hsp90 IgM was the most frequent in contrast to the childhood SLE group in which IgG anti-hsp 90 was the most frequent.

The clinical characteristics of the patients with JCA positive for anti-hsp antibodies are shown in table 4.3. There were no differences between the
Table 4.3 Clinical characteristics of JCA patients with HSP 70/90 antibodies.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Pt.</th>
<th>JCA subtype</th>
<th>Sex</th>
<th>Age</th>
<th>Dur*</th>
<th>Act°</th>
<th>Infection</th>
<th>ANA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP 70 IgG</td>
<td>1</td>
<td>Sero-poly</td>
<td>M</td>
<td>6.5</td>
<td>2</td>
<td>1</td>
<td>URI</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Systemic</td>
<td>M</td>
<td>10.5</td>
<td>7</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Pauci</td>
<td>F</td>
<td>5</td>
<td>0.2</td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IgM</td>
<td>4</td>
<td>Sero-poly</td>
<td>F</td>
<td>14</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Pauci-poly</td>
<td>F</td>
<td>6.5</td>
<td>4</td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Sero-poly</td>
<td>F</td>
<td>9.5</td>
<td>6.5</td>
<td>3</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HSP 90 IgG</td>
<td>7</td>
<td>Pauci</td>
<td>F</td>
<td>3.5</td>
<td>0.3</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Pauci-poly</td>
<td>F</td>
<td>18</td>
<td>16</td>
<td>2</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>IgM</td>
<td>9</td>
<td>Sero-poly</td>
<td>F</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Sero-poly</td>
<td>F</td>
<td>15</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Pauci</td>
<td>F</td>
<td>12</td>
<td>0.2</td>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Pauci</td>
<td>F</td>
<td>7.5</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Sero-poly</td>
<td>F</td>
<td>11.5</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* = Disease duration at time of serum sample, in years.
° = Presence of infection within 3 months of serum sample.
group as a whole with respect to age, sex or disease duration compared to JCA patients positive for anti-hsp reactivity; however, three of the five patients with pauci-articular onset progressing to a polyarticular course had hsp reactivity. The majority of patients with anti-hsp reactivity had moderate-severe disease activity at the time of testing, with only 2/13 having mild disease at the time.

Synovial Fluid

These results are shown in table 4.1. Six out of the 12 samples (50%) had IgG antibodies to hsp70. No IgM antibodies to hsp70 were found nor were antibodies to hsp90 of either isotype. In addition the mean level of anti-hsp70 antibody of this group was the highest of all patient groups tested. Of the positive samples two were from patients with polyarticular JCA and four had pauci-articular JCA.

Antibodies to hsp90 and 70 in patients with juvenile dermatomyositis

These results are shown in Table 4.1. Four out of 12 patients are positive for IgG antibodies to hsp70; one is positive for IgM antibodies to hsp70. Three out of 12 patients are positive for IgG antibodies to hsp90 but no IgM antibodies were detected.

Table 4.4 compares the percentage of adult and juvenile patients with elevated levels of antibodies to hsp90 and hsp70. The table shows that the percentage of patients with SLE with elevated IgG anti-hsp90 antibodies is very similar in both the adult and juvenile patients, unlike the percentage of patients with increased expression of IgM anti-hsp90 antibodies which is far
Table 4.4 A comparison of the percentages of patients (juvenile and adult onset) with autoimmune rheumatic diseases with elevated levels of anti-hsp90 and anti-hsp70 antibodies.

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>JSLE</th>
<th>RA</th>
<th>JRA</th>
<th>DM</th>
<th>JDM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-hsp90 antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>26</td>
<td>23.5</td>
<td>0</td>
<td>3.6</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>IgM</td>
<td>34</td>
<td>3</td>
<td>7</td>
<td>9</td>
<td>33</td>
<td>5.2</td>
</tr>
<tr>
<td><strong>Anti-hsp70 antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>8.9</td>
<td>8.8</td>
<td>13</td>
<td>5.4</td>
<td>NT</td>
<td>30</td>
</tr>
<tr>
<td>IgM</td>
<td>7.1</td>
<td>5.9</td>
<td>6</td>
<td>5.4</td>
<td>NT</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Results recorded as percentage of patients with elevated levels of antibody

JSLE Patients with Juvenile onset SLE
JCA Patients with Juvenile chronic arthritis
JDM Patients with Juvenile dermatomyositis

NT=not tested
higher in the adult patients with SLE compared to the juvenile patients. The percentage of patients with raised levels of IgG or IgM anti-hsp70 antibodies is very similar in both the adult and juvenile patients with SLE. IgG and IgM anti-hsp90 antibodies were found at similar frequency in adult and juvenile forms of rheumatoid arthritis. The percentage of patients with dermatomyositis with elevated IgG anti-hsp90 antibodies is very similar in both the adult and juvenile patients, unlike the percentage of patients with increased expression of IgM anti-hsp90 antibodies which is far higher in the adult patients with myositis compared to the juvenile patients.

4.3. Discussion

Having demonstrated (chapter 3) that anti-hsp90 antibodies are elevated in adult patients with SLE and myositis, the study was then extended to children with rheumatic diseases. Previous reports have demonstrated cellular and humoral reactivity to bacterial hsps such as hsp60 and 70 in children with juvenile chronic arthritis [Life et al. 1993, Schulz and Arnold 1993, Albani et al. 1994] but there have been no previous studies testing for antibodies to native purified mammalian hsp90 in children with SLE or other autoimmune rheumatic diseases.

In contrast to adult patients with SLE, antibodies reactive with hsp90 and/or 70 were found in 35% of the children with SLE, IgG anti-hsp90 antibodies were detected in 23.5% of the juvenile patients with SLE, whilst only 3% had elevated IgM anti-hsp90 antibodies. Fewer patients demonstrated reactivity with hsp70; 8.8% had IgG anti-hsp reactivity and 5.8% had IgM anti-hsp70 reactivity. A previous study detected antibodies to denatured hsp73 in two
out of 16 (12.5%) of patients with juvenile SLE. The study omits whether these anti-hsp73 antibodies are of the IgG or IgM isotype [Minota et al. 1988], and does not relate antibodies to disease activity. In our study there was no significant difference in age or disease duration between those patients with antibodies to hsp90 or 70 and those without hsp reactivity. The patients with anti-hsp90 antibodies showed an increase in renal disease whereas there were no differences in joint involvement, rash, cardiopulmonary disease or central nervous system disease. This is similar to the situation found in the adult patients with SLE. There were no differences in abnormal serology at the time of sampling between the positive and negative groups. The patients who were positive for anti-hsp antibodies were more likely to have had an infection within three months of serum sampling than those who were antibody negative, the infections seen were sinusitis, urinary tract infection and salmonella septicaemia. Thus, this might imply that these antibodies could result from infection. None of the control group had an infection at time sera was taken.

It would be of interest to obtain sera from younger patients (six to twelve months) and relate hsp90 and hsp70 antibodies to vaccination status. It has been demonstrated that infants immunised with the trivalent vaccine against diphtheria, tetanus and pertussis (DTP), develop an antibody response to mycobacterial hsp60, and these antibodies will also bind to human hsp60, albeit at much lower levels [Del Guidice et al. 1993]. The authors suggest that priming of the immune system is a common phenomenon occurring early in life. These results might explain why some T cells in apparently healthy individuals can react with mycobacterial hsp [Munk et al. 1989].

It is notable that the percentage of adult and childhood onset SLE patients
with elevated IgG anti-hsp90 levels is almost identical (26% vs. 24%). However, there was a marked difference in the prevalence of IgM isotype anti-hsp90 antibodies between the adults and children, with IgM more prevalent in the former (35%) compared to the latter (3%). The relative lack of overexpression of anti-hsp70 antibodies (less than 9%) in these patients supports the view that the anti-hsp90 response is not a result of a nonspecific reaction to hsps.

Given that the first antibody isotype to become detectable following infection is IgM, it is interesting that of the patient populations studied to date, it is the paediatric SLE patients in whom a potential link with infection has been noted, compared to the situation with adult patients with SLE. However the total number of children studied is relatively small and no firm conclusions can be drawn at present. Even if this correlation is confirmed with a larger sample size, the overexpression of anti-hsp90 antibodies of the IgG isotype might be linked to underlying disease expression, and possibly activity (notably in those patients with renal involvement), rather than being the consequence of recent infection.

This data implies that over-expression of anti-hsp90 antibodies in SLE is not merely part of a random nonspecific reaction to all hsps in autoimmune rheumatic diseases. The data showing that reactivity to hsp90 in childhood onset SLE is similar to that in adult onset disease suggests that despite clinical differences between these two groups at least a subset of each could share similar pathological mechanisms of disease.

Antibodies to hsp 70 and/or 90 were found in 23.6% of patients with JCA. Anti-hsp90 IgM antibodies were the most frequently detected anti-hsp90
isotype in patients with JCA, whereas IgG anti-hsp90 was the most frequent in juvenile patients with SLE. There were no differences from the group as a whole with respect to age, sex or disease duration compared to the JCA patients positive for hsp reactivity. The majority of patients with anti-hsp reactivity had moderate-severe disease activity at the time of testing, with only 2/13 having mild disease at the time.

Synovial fluid was obtained from 12 patients with JCA, although these were not the same patients from whom sera were obtained. IgG antibodies to hsp70 were detected in 50% of these samples. No IgM antibodies to hsp70 were found nor were antibodies to hsp90 of either subclass. Of the positive samples two were from patients with polyarticular JCA and four had pauciarticular.

IgG antibodies to hsp70 were detected in four out of 12 patients with myositis, one was positive for IgM antibodies to hsp70. Three out of 12 patients had elevated levels of IgG anti-hsp90 antibodies, but no IgM antibodies were detected. These results parallel the findings in patients with juvenile and adult-onset SLE. It would be interesting to increase the sample size of this patient group and obtain clinical data about these patients.

The low frequency of reactivity to hsps among control children without rheumatic conditions is important, and suggests specificity of this response to patients with autoimmune disorders.

Children with JCA have been shown to have an increased frequency of immune responses to hsps of the 60kD, 65kD and 70kD families [Schulz and Arnold 1993], notably human and mycobacterial hsp65 [Nualláin et al. 1993],
E. coli dnaJ hsp [Albani et al. 1994], and human hsp60 [De Graeff-Meeder et al. 1993]. In the last of these studies reactivity with hsp60 was found to be elevated in synovial fluid of patients with JCA compared to simultaneously obtained serum samples, and an increased T lymphocyte proliferative response to human hsp60 has been reported in cells from the synovial fluid and peripheral blood [De Graeff-Meeder et al. 1991]. Although most previous studies have not examined isotype differences in hsp reactivity in children with JCA, one study [Nualláin et al. 1991] did report that 30% and 27% of 56 JCA patients had IgM and IgG antibodies respectively to (mycobacterial) hsp65. In this study it was also reported that 7.1% and 17.8% of the patients tested had raised IgG and IgM antibody levels to human hsp70, which compares to 5.4% of both isotypes we report in this study. In contrast 34% of patients reported by Nualláin et al. [1991] had an IgA response to human hsp70. IgA anti-hsp antibodies were not tested in these patients.

IgG and IgM antibodies to hsp70 and 90 were found at a similar frequency (less than 9%) in children with JCA and in the adults with rheumatoid arthritis. Among the JCA patients IgM anti hsp90 was the most frequently seen, with little evidence of an IgG response to hsp70 or 90. These initial results might imply that it is unlikely that hsps are of clinical relevance in this disease. However, since 50% of the synovial samples tested had detectable levels of IgG anti-hsp70 antibodies, albeit in a limited sample size, hsp70 may well be involved in the pathogenesis of RA. It would be of interest to increase the number of synovial fluid samples as well as paired sera and synovial fluid samples from both patients with JCA and RA. Evidence in support of involvement of hsp70 in RA comes from recent work
demonstrating that there are increased numbers of secretory B cells to hsp70 from the synovium from patients with RA [Winfield 1994].

Thus it appears that there is differential expression of hsps in patients with SLE and in other autoimmune diseases both at the protein level and antibody level. Therefore increased expression of hsps and antibodies to them are not simply part of a generalised stress response to autoimmune disease.
5.1 Antibodies to hsps in patients with cancer

The successful development of an ELISA to detect antibodies to hsp90 enabled rapid testing of a variety of sera for the presence of anti-hsp90 antibodies. As described in section 1.2, levels of hsp90α expression had been measured in patients with breast cancer and shown to be elevated in patients with malignant breast cancer compared to patients with benign breast cancer [Jameel et al. 1989]. Therefore, the opportunity arose to test sera from patients with breast cancer and other types of cancer for anti-hsp90 antibodies and to quantitate these results enabling comparisons to be made between the different diseases. Although protein levels had been measured in breast tissue from patients with breast cancer, it was not possible to obtain serum samples from the same patients for whom protein data was available. Initially a serum sample was obtained from 71 patients with breast cancer, prior to initial surgery and not currently undergoing any treatment, and tested for antibodies to hsp90. The levels were then compared to those found in SLE and normal healthy controls (fig 5.1). These results show elevated levels of IgG anti-hsp90 antibodies in patients with breast cancer compared to healthy controls.

All of these anti-hsp90 antibodies detected in the breast cancer group were IgG; in comparison to the situation in SLE where IgM was the more frequently detected isotype. IgM anti-hsp90 antibodies were not detected in sera from
Fig 5.1 IgG antibodies to hsp90 in patients with breast cancer, SLE, and healthy controls.

Fig 5.2 IgG antibodies to hsp90 in patients with malignant breast cancer compared to benign breast cancer.
Table 5.1 Comparison of IgG anti-hsp90 antibodies in patients with malignant breast cancer, benign breast cancer, SLE and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Malig B.Cancer</th>
<th>SLE</th>
<th>Healthy Controls</th>
<th>Benign B. Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients tested</td>
<td>70</td>
<td>72</td>
<td>28</td>
<td>26</td>
</tr>
<tr>
<td>No. with +ve Abs</td>
<td>18</td>
<td>19</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mean of total group</td>
<td>8</td>
<td>4.2</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Mean of +ve group</td>
<td>31.2</td>
<td>13.2</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

+ve refers to the those values of IgG antibodies that were elevated i.e greater than the mean +2SD of the healthy controls.
any of these patients with breast cancer. The means of the SLE group were then compared to the means of the breast cancer group, benign breast cancer group and normal healthy controls (table 5.1). It is apparent that there is little difference between the number of patients with elevated IgG anti-hsp90 antibodies in patients with SLE and in patients with breast cancer; although the overall mean level of antibodies and the mean of just the positive samples is much higher in the breast cancer group than in the SLE samples. Sera from 26 patients with benign breast tumours were also tested for IgG and IgM anti-hsp90 antibodies (fig 5.2). None of these patients had elevated levels of anti-hsp90 antibodies. These results indicate that it is the patients with malignant breast cancer rather than those with benign breast tumours, who have these antibodies to hsp90, and unlike in adult-onset SLE, a general autoimmune disease, these antibodies are only of the IgG isotype.

The number of breast cancer samples studied was then increased to 126 and serum samples from patients with other types of cancer were tested. These results are shown in fig 5.3. 46 (37%) of breast cancer patients showed high levels of IgG antibodies (greater that the mean plus three standard deviations observed in sex-matched normal controls), whereas this was not observed in normal individuals and patients with benign breast tumours and relatively rarely in patients with other forms of tumours. It is the breast cancer serum samples that have the highest levels of anti-hsp90 antibodies, although these antibodies can be detected (albeit at low levels) in some patients with ovarian and prostate cancer.

Benign and malignant breast cancer samples were then tested for antibodies to hsp70 and hsp27 (table 5.2). Increased hsp27 staining has been shown to
Fig 5.3 IgG antibodies to hsp90 in patients with breast cancer, other cancers, benign tumours and SLE.
be associated with shorter disease free survival but longer time to initial
relapse in patients without nodal involvement [Love and King 1994]. An
ELISA was established to measure antibodies to hsp27 as described in
methods section. Antibodies to hsp70 and hsp27 were detected in patients
with breast cancer but not in patients with benign tumours (table 5.2).
However it was only anti-hsp90 antibodies that were shown to be statistically
significantly elevated in patients with breast cancer compared to benign
samples, probably due to small numbers of patients with elevated levels of
anti-hsp70 and anti-hsp27 antibodies.

Anti-nuclear antibodies (ANA) were then tested in 20 sera samples that had
the highest levels of anti-hsp90 antibodies, courtesy of Marlene Swanna
(Department of Immunology, University College London Medical School).
None of these samples were positive for ANA suggesting that the presence
of these anti-hsp90 antibodies is not part of a generalised immune response
which may be the situation in patients with SLE and other autoimmune
diseases.

5.2 Clinical Associations of anti-hsp90 antibodies in patients
with breast cancer

5.2.1 Estrogen Receptor Status
Estrogen receptor status data was available in only 25 out of the 123 patients
with breast cancer for whom any clinical data was available. The results are
summarised in table 5.3. The numbers are too small to draw any
conclusions.
Table 5.2. The number of patients with elevated levels of IgG anti-hsp90 antibodies, anti-hsp70 antibodies and anti-hsp27 antibodies in patients with malignant and benign breast tumours

<table>
<thead>
<tr>
<th>Antibody Tested</th>
<th>Statistically Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Anti-hsp27 antibodies</td>
<td></td>
</tr>
<tr>
<td>Ab +ve</td>
<td>Ab-ve</td>
</tr>
<tr>
<td>Malig.</td>
<td>4</td>
</tr>
<tr>
<td>Benign</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N/S</td>
</tr>
<tr>
<td>2. Anti-hsp70 antibodies</td>
<td></td>
</tr>
<tr>
<td>Ab +ve</td>
<td>Ab-ve</td>
</tr>
<tr>
<td>Malig.</td>
<td>8</td>
</tr>
<tr>
<td>Benign</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N/S</td>
</tr>
<tr>
<td>3. Anti-hsp90 antibodies</td>
<td></td>
</tr>
<tr>
<td>Ab +ve</td>
<td>Ab-ve</td>
</tr>
<tr>
<td>Malig.</td>
<td>18</td>
</tr>
<tr>
<td>Benign</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Significant</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

NS not significant
Table 5.3 Clinical Analyses of IgG anti-hsp90 antibodies in patients with breast cancer.

<table>
<thead>
<tr>
<th>Statistically Significant</th>
</tr>
</thead>
</table>

1. Estrogen receptor. Levels measured 25 out of 123 pts.

<table>
<thead>
<tr>
<th></th>
<th>ER+ve</th>
<th>ER-ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp90+ve</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Hsp90-ve</td>
<td>13</td>
<td>4</td>
</tr>
</tbody>
</table>

2. Nodal Status

<table>
<thead>
<tr>
<th></th>
<th>Nodes +ve</th>
<th>Nodes-ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp90+ve</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>Hsp90-ve</td>
<td>27</td>
<td>19</td>
</tr>
</tbody>
</table>

NS not significant
5.22 Axillary Lymph Node Metastasis (Nodal Involvement)
Axillary lymph node metastasis (nodal involvement) was assessed in 73 patients. Patients were considered to be positive if the cancer had spread to more than one node. The results for this analysis are summarised in table 5.3. There may be a tentative relationship in that there were far fewer patients that had both elevated levels of anti-hsp90 antibodies and involved nodes, although the numbers failed to reach statistical significance.

5.23 Type of Carcinoma
These results are summarised in table 5.4. There did not appear to be any apparent correlation between type of carcinoma and the presence of raised levels of anti-hsp90 antibodies, although it is apparent that elevated levels of these antibodies are detected in both invasive and non-invasive carcinomas.

5.24 Tumour Grade
Clinical parameters between patients with different tumour grades, which reflect the degree of differentiation of the tumour were investigated related to the patient having elevated levels of anti-hsp90 antibodies. These results are summarised in table 5.5. There did not appear to any apparent correlation between tumour grade and anti-hsp90 antibodies, although very few patients had had tumour grade 1 and none were reported as having a tumour grade 4.

5.25 Metastases
Multi-variate pairwise linear regression on the first 67 breast cancer patients (for whom clinical follow up data was available) indicated that the presence of autoantibodies to hsp90 \( (p<0.04) \) and the presence of involved nodes \( (p<0.001) \) were both statistically significantly correlated with the development
Table 5.4 Analysis of type of carcinoma in patients with breast cancer related to IgG anti-hsp90 antibody status

<table>
<thead>
<tr>
<th>Type of carcinoma</th>
<th>Hsp90+ve</th>
<th>Hsp90-ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDC</td>
<td>22</td>
<td>53</td>
</tr>
<tr>
<td>Lob</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>DCIS</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>IDC + DCIS</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>IDC + Lob</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Others</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

IDC = Infiltrating ductal carcinoma
Lob = Lobular carcinoma
DCIS = Ductal carcinoma in situ

Table 5.5 Analysis tumour grade in patients with breast cancer related to IgG anti-hsp90 antibody status

<table>
<thead>
<tr>
<th>Grade</th>
<th>Hsp90+ve</th>
<th>Hsp90-ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
of metastases. Table 5.6 shows that 28% of patients with antibodies to hsp90 developed metastases compared to 12% of patients without antibodies. The presence of antibodies to hsp90 and positive nodes together, were more closely correlated with the development of subsequent metastatic recurrence than nodal involvement alone, with no patients with metastases lacking both nodal involvement and antibodies to hsp90 (Table 5.6). The correlation was also significant when tumour size, tumour grade and age of diagnosis were included in the regression equation (personal communication Stephen Gibson).

Clinical data then became available for the second set of patients, although there was a significant difference in follow-up time for patients within the second group. The mean follow up time for the first 67 patients was approximately 4.7 years compared to 3.3 years in the second group. Table 5.7 shows that 22% of patients with antibodies to hsp90 developed metastases compared to 9% of patients without antibodies. The analysis of all patients with metastasis is shown in fig 5.4. There are no patients who are positive for both antibodies to hsp90 and nodal involvement at time of initial surgery who have gone on to develop metastasis. In comparison 45% of the patients who have not developed metastasis were negative for both antibodies to hsp90 and only 4% of the total patients (n=123) studied without metastasis were positive for both nodal involvement and elevated antibodies to hsp90 (fig 5.5). These patients were from the second group of patients with a much shorter follow up time. In the first group of patients (n=67), there were no patients without metastasis who were positive for both nodal involvement and raised antibodies to hsp90 (fig. 5.5)
Table 5.6. Analysis of the presence of metastasis with IgG antibodies to hsp90 in the first 67 patients with breast cancer

<table>
<thead>
<tr>
<th></th>
<th>Metastasis</th>
<th>No Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp90 -ve.</td>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>Hsp90 +ve</td>
<td>5</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 5.7. Analysis of the presence of metastasis with antibodies to hsp90 and nodal involvement in the first set of patients with breast cancer in whom nodal status was known.

<table>
<thead>
<tr>
<th></th>
<th>Metastasis</th>
<th>No Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp90 and nodes -ve</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Hsp90 and nodes +ve</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>Hsp90 +ve, nodes -ve</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Hsp90 -and nodes +ve</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td><strong>p&lt;0.05</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.8. Analysis of the presence of metastasis with antibodies to hsp90 in 118 patients with breast cancer

<table>
<thead>
<tr>
<th></th>
<th>Metastasis</th>
<th>No Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp90 -ve.</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>Hsp90 +ve</td>
<td>9</td>
<td>32</td>
</tr>
</tbody>
</table>
Fig: 5.4 A comparison of the incidence of nodal involvement and IgG antibodies to hsp90 in patients with and without metastasis.

Outer Circle: All Patients with Metastasis
Inner Circle: All Patients without Metastasis

H  IgG antibodies to hsp90
N  Nodal involvement
+ve Present
-ve Absent
Fig: 5.5 The incidence of IgG antibodies to hsp90 and nodal involvement in patients without metastasis. A comparison of the initial long term sample and all patients.

H  IgG antibodies to hsp90
N  Nodal involvement
+ve Present
-ve Absent

Outer Circle: All Patients without Metastatis
Inner Circle: Initial Group of Patients without Metastatis
who were negative for both antibodies to hsp90 and nodal involvement.

The presence of antibodies to hsp90 and hsp70 were found to correlate with each other (p<0.01), although there was no correlation between anti-hsp70 antibodies, nodal involvement and the development of metastases. No correlation was found between the presence of hsp90 antibodies and the presence of anti-hsp27 antibodies, nor was there any correlation between the presence of anti-hsp27 antibodies and anti-hsp70 antibodies. A correlation was found between patients who had anti-hsp27 antibodies and a high tumour grade (p<0.0007).

5.3 Discussion

These results demonstrate that antibodies that recognise human hsp90, can be detected in the sera of patients with breast cancer at higher frequency and at higher titres than in healthy controls and patients with benign breast tumours. These results might imply that hsp90 has some involvement in the development of malignant but not benign breast cancer.

In these assays 37% of the patients showed high levels of anti-hsp90 antibodies whereas this was not observed in normal individuals or patients with benign breast tumours and only very rarely in patients with other forms of tumours. In general, the levels of autoantibodies to hsp90 detected in the breast cancer patients were higher than those observed in SLE patients and occurred in a greater proportion of the patients. In addition these antibodies were of the IgG subclass and not of the IgM subclass more commonly found in patients with autoimmune diseases, thus suggesting that this response might be antigen driven rather than reflecting a generalised autoimmune
response. In agreement with this no antibodies to nuclear proteins (ANA) which are common in autoimmune disease were detected when sera from 25 breast cancer patients positive for antibodies to hsp90 were tested indicating that the elevation in antibodies to hsp90 is a specific effect.

5.31 Possible Mechanisms resulting in the production of anti-hsp90 antibodies in patients with breast cancer

It has been demonstrated that inbred mice and rats immunised against their own tumours or tumours of the same genetic background become immune to challenges with tumour cells [Srivastava and Old 1988]. These studies demonstrated that mice vaccinated with inactive cancer cells become immune to subsequent challenges with live cancer cells. This response was tumour specific in that mice became immune to the tumours that were used to immunise them and not to other tumours.

This led to the concept of immunogenicity, and the search for cancer derived molecules which elicited resistance to tumour challenges. The general approach used was to take fractionated cancer cell derived proteins and test them individually for their ability to immunise mice against the cancers from which the fractions were prepared. A number of proteins have been identified using this approach and a large proportion of these were found to be related to the hsp9s. Given that these proteins are amongst the most highly conserved proteins between species throughout evolution, it is unlikely that they are tumour specific antigens. Hsp9s isolated from healthy tissues did not elicit immunity against any tumours tested i.e. there did not appear to be any cross immunity. There was no tumour cross protection, the mice could only be immunised against the tumour from which the peptides were extracted.
These observations have now been made with three distinct hsps: gp96, hsp90 and hsp70 and would appear to suggest the existence of tumour-specific differences in all three. However comparison of cDNA sequences of gp96 and hsp90 from healthy tissue and antigenically distinct tumours did not reveal any differences in DNA sequences [Srivastava et al. 1991].

As some hsps can bind a diverse array of molecules including peptides it is possible that it is not the hsps themselves that are immunogenic but the peptides which are transported by the hsps. This theory was tested and found to be correct [Udono and Srivastava 1993]. Udono and Srivastava vaccinated mice with hsp70 preparations derived from MethA sarcoma and found that this renders the mice immune to a subsequent challenge with MethA sarcoma. The immunogenicity was found to be dose dependent and tumour specific. When the antigenically active hsp70 was further purified with ATP affinity chromatography the purified intact hsp70 remaining was no longer able to render the mice immune to subsequent challenges. Separation of the low molecular weight material showed a diverse range of peptides with molecular masses between 1000-5000 daltons. These results suggest that the antigenicity derives, not from hsp70 per se but from associated peptides. The authors conclude that the peptides are derived from cellular proteins by proteolytic degradation. The authors postulate that the repertoire of peptides generated in the tumour cells is likely to differ from those generated in normal tissues because of tumour-associated mutations, which would explain the difference in antigenicity of tumour compared to normal tissue derived hsp70. It is not clear whether the peptide-binding activity is found in all subsets of the hsp70 family.
Recently, there has been much evidence to indicate that hsp90, gp96 and hsp70 associate with antigenic peptides derived from cellular proteins. This has led to two hypotheses being proposed: 1) that hsps constitute a relay line in which the peptides, after generation in the cytoplasm by proteases, are transferred from one hsp to another, until they are finally accepted by MHC class 1 molecules in the endoplasmic reticulum and 2) that the binding of peptides by hsps constitutes a key step in the priming of cytotoxic T lymphocytes (CTLs) in vivo. One possibility is the following: hsps are released from tumour cells in vivo during lysis of cells through infection or by the action of antibodies. The hsps which are now complexed with antigenic peptides derived from cognate cells are taken up by macrophages or other specialised antigen-presenting cells. The hsp-borne peptide is then routed to the endogenous presentation pathway in the antigen-presenting cell and is displayed in the context of that cell's MHC class I, where it is finally recognised by the precursor CTLs. This mechanism explains the phenomenon of cross-priming and has implications for the development of immunological strategies against cancer.

Therefore one explanation for the presence of anti-hsp90 antibodies in patients with breast cancer might be that the hsp90 is transporting peptides on to the cell surface leading to the generation of antibodies against them. Another explanation for the presence of antibodies to hsp90 being detected at higher frequency in those patients who were more likely to go on to develop metastasis might be that more cells are transporting peptides of hsp90 to the cell surface. Alternatively it is possible that the movement of the cancer cell from the breast to site of metastases results in exposure of the antigen to the immune system, which might explain why the antibodies were
found in patients who subsequently went on to exhibit metastases.
Concluding Comments

The initial aim of this work was to develop a simple, and accurate method for detecting antibodies to human hsp90 in order to investigate the possibility that antibodies might arise, possibly from overexpression of the protein and translocation of hsp90 or some component of it on to the cell surface in patients with SLE.

A suitable ELISA was established using purified native human hsp90. IgG and or IgM anti-hsp90 antibodies were over expressed in almost a third of patients with SLE compared to healthy controls and patients with most other autoimmune rheumatic diseases. The antibodies showed little clinical correlation except with active renal disease and previous bacterial infection. It was interesting that those patients who were A1/B8/Dr3 negative were most likely to overexpress these antibodies. With one exception, all patients with an infection and elevated antibodies to hsp90 at the time of serum collection were, also of this HLA type. These results suggest a possible a genetic predisposition in patients with SLE to develop these antibodies, possibly triggered by some type of infection. It would be of interest to increase this sample size of patients with SLE with an infection and compare levels of antibodies to those levels found in normal healthy controls.

Given that antibodies to hsp70 were expressed at similar levels in patients with SLE and healthy controls this might indicated that these antibodies are not simply a generalised stress response, rather a specific response to hsp90. Even if the initial trigger for the production of these antibodies is bacterial hsp90, the antibodies can recognise human hsp90 implying that
molecular mimicry could occur.

Epitope mapping, with sera from a range of patients with infections, revealed that antibodies commonly produced to hsp90 cross-react with a number of epitopes on hsp90 whereas sera from patients with SLE recognise different epitopes of hsp90 [Al-Dughaym et al. 1994]. It would be interesting to use epitope mapping with human hsp90 to investigate whether the epitopes recognised by antibodies produced by patients with SLE are the same as those epitopes recognised by patients with breast cancer.

It was fascinating to discover that patients with malignant breast cancer had higher levels and increased frequency of antibodies to hsp90 than healthy controls and patients with benign breast tumours. Elevated levels of these antibodies appears to be a significant indicator of the presence of breast cancer metastases. The measurement of such autoantibodies could thus become a valuable additional indicator to aid in post surgical therapeutic management decisions; particularly since such antibodies can be readily measured in routine serum samples. Further studies, using a larger patient population are currently being undertaken to investigate this possibility and to indicate the usefulness of analysing serial samples to monitor for disease recurrence.

This study indicates several further interesting areas of research. Firstly, to obtain breast tumour material to quantify hsp90 protein levels as well as look at surface expression of hsp90 on the tumour in these patients and relate this to the antibody data. Secondly, to subclass the antibody response in patients with SLE and breast cancer who overexpress the IgG isotype of hsp90 antibodies and carry out inhibition assays using bacterial hsp90.
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Appendix I

1. Tissue Culture

All the tissues culture work was carried out in a Gelaine Class II hood which was regularly cleaned using hypochlorite and exposed to formalin every six months. Immediately prior to and straight after use the surface was cleaned with 70% alcohol. The cells were maintained in a Heraeus incubator which was maintained at 37oC with 5% carbon dioxide in a water saturated atmosphere. The incubator was cleaned regularly and copper sulphate was placed in the water at the base of the incubator to reduce risk of fungal infection. The CBF7 cell line was screened every six months for mycoplasma infection (courtesy of the ICRF at UCL).

Tissue Culture medium

Tissue culture medium was made in batches of 500ml as required. Prior to use 10ml was taken placed on a petri dish for 48hrs to check for the presence of infection.

Growth Medium

All these reagents were obtained from Gibco, Paisley, Scotland

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640</td>
<td>83.8%</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>10%</td>
</tr>
<tr>
<td>MEM non-essential amino acids (100x)</td>
<td>2%</td>
</tr>
<tr>
<td>L-glutamine 200mM (100x)</td>
<td>1%</td>
</tr>
<tr>
<td>HEPES 1M</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium pyruvate 100mM</td>
<td>1%</td>
</tr>
<tr>
<td>Penicillin (10000 IU/ml) and</td>
<td></td>
</tr>
<tr>
<td>Streptomycin (10000µg/ml)</td>
<td>1%</td>
</tr>
<tr>
<td>Gentamicin (10mg/ml)</td>
<td>0.2%</td>
</tr>
</tbody>
</table>
HAT/HT Medium

The HAT/HT and insulin were obtained from Sigma (Poole Dorset). The remaining reagents listed were obtained from Gibco.

RPMI 1640 60.8%
Fetal calf serum 20%
NCTC-135 with L-glutamine 10%
MEM non-essential amino acids (100x) 2%
HAT or HT x50 2%
Insulin solution 1%
Sodium pyruvate 100mM 1%
Penicillin (10000 IU/ml) and Streptomycin (10000μg/ml) 1%
Gentamicin (10mg/ml) 0.2%

Preparation of HAT/HT solution
One vial of HAT/HT (Sigma) was diluted in 10ml RPMI to give the following concentrations: HAT, Hypoxanthine 5x10^-3 M; Aminopterin 2x10^-5M; Thymidine 8x10^-4M; HT: Hypoxanthine 5x10^-3 M; Thymidine 8x10^-4M. The post fusion medium is identical to the HAT medium but without the HAT.

Serum free Medium

SF-1 was obtained from Northumbria Biologicals. The remaining reagents were obtained from Gibco

SF-1 97.8%
L-glutamine 1%
Penicillin (10000 IU/ml) and Streptomycin (10000μg/ml) 1%
Gentamicin (10mg/ml) 0.2%

Storage of cell lines

The cells were washed twice using RPMI medium, counted, spun down and
then resuspended in 50% fetal calf serum, 30% RPMI and 20% sterile dimethylsulfoxide (Analar, BDH, Poole, UK) at a density of $10^7$/ml. The cell suspension was aliquoted into freezing tubes (Nunc cryotubes 1.8ml) and placed in a Naglene Cryo $1^\circ$C freezing container in a -80$^\circ$C freezer for a minimum of 4hrs in order to achieve a cooling rate of $1^\circ$C/min. using proan-2-ol (BDH). The tubes were then placed in a liquid nitrogen tank for long term storage.

**Buffers**

**1 ELISA Solutions**

**Bicarbonate Buffer:** Sodium bicarbonate 1.55g  
Sodium carbonate 0.8g  
Dissolved up to 1 litre pH 9.5

**Phosphate buffered saline (PBS)**  
To prepare 10 litres of 10xPBS  
KCL 20g  
KH$_2$PO$_4$ 20g  
Na$_2$HPO$_4$ 114.8g  
NaCl 800g  
This was then dissolved, pH adjusted to 7.4 with 5M NaOH and then diluted tenfold with distilled water. The pH was then rechecked before use.

**PBS-T.**  
This was prepared in the same way as PBS with the addition of 0.05% Tween20 (Sigma)

**Preparation of DNA for the DNA ELISA**

A stock solution of calf thymus DNA (Sigma) of 1mg/ml was dissolved in distilled water overnight and sonicated in ice. To prepare single stranded DNA this solution was diluted to 500$\mu$g/ml and boiled for 10 min before
cooling on ice for 15 min. Double stranded DNA was prepared by adding an equal volume of DNA stock solution to S1 nuclease solution (1U/ml) immediately prior to use for 1 hour at 37°C.

**Protein Electrophoresis and Western Blotting**

**Sample buffer:**
2.3% sodium dodecyl sulphate (SDS)
0.0625M Tris, 10% glycerol,
5% β-mercaptoethanol bromophenol-blue.

**Lower gel:**
7% acrylamide in 1.5M Tris, 0.4% SDS, pH 8.8

**Stacking gel:**
5% acrylamide in 0.5M Tris, 0.4% SDS, pH 6.8

**Running buffer:**
0.192M glycine, 0.025M Tris, 0.1% SDS, pH 8.3

**Circulating buffer:**
0.192M Tris, 0.025M Glycine, 0.025M Tris, 20% Methanol

**pH 8**

**Blocking buffer:**
10% marvel, Tris-buffered saline (TBS) and 0.05% Tween-20

**Washing buffer:**
0.3% Marvel, TBS, 0.05% Tween-20

**Coomassie Blue**
1.06g Coomassie sol'n, 7% acetic acid, 50% methanol

**Destain**
10% acetic acid, 50% methanol, 40% water
Appendix II

BILAG SCORING SYSTEM

The following is a copy of the guidance notes for completing a BILAG assessment.

Assumption
It is implicit in the scoring system that all features scored are thought to be due to active lupus.

The questionnaire asks whether features are improving, the same, worse or new during the last month, or since the last assessment if this was performed less than one month ago. If a new feature has developed in the last month (or since the last assessment if less than a month ago) it should be scored as new (ie 4), even if it has subsequently improved or resolved. For the first assessment any response will register the feature as a criterion. For subsequent assessments, features will only contribute to the score if they are the same, worse or new. These different grades have been used so that BILAG can identify all patients who have developed a particular feature for the first time and also to document the response of particular features to treatment.

In the renal and haematological assessments (which include laboratory tests) the assessor will be asked for confirmation that abnormal results are due to active lupus (rather than drug side effects for example).

When going through the individual screens for the first time it may be that no data has been entered for a particular screen. If this happens the patient will be assigned to either a category E or D for that particular system. If the patient has ever had any involvement of that system D should be entered and E if there has never been previous involvement. Once a patient has scored an A, B, C or D in a particular system she or he will always score at least a D in the future. The score E implies no involvement of the system ever.
1. GENERAL NON-SPECIFIC MANIFESTATIONS (Gen)

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

**Category A**
Pyrexia

**Category B**
Pyrexia or two others

**Category C**
Any other one criterion

**Category D**
Previous involvement

**Category E**
No involvement

**Mucocutaneous Disease (Muc)**

**Category A-** any one of
1. Severe maculopapular, discoid or bullous eruption; ie active facial and/or extensive (>2/9), scarring or causing disability.
2. Angio-oedema
3. Extensive mucosal eruption
4. Severe active alopecia
5. Subcutaneous nodules
6. Perniotic skin lesions

**Category B -** any one of
1. Malar erythema
2. Mild maculopapular eruption
3. Panniculitis
4. Localised active discoid lesions incl. lupus profundus

**Category C-** any one of
1. Periungal erythema
2. Swollen fingers
3. Sclerodactyly
4. Calcinosis
5. Telangiectasia
6. Mild alopecia
7. Small mucosal ulceration

**Category D**
Previous involvement

**Category E**
No involvement
CNS disease attributable to lupus (CNS)
First assessment

Category A
Acute, progressive or recurring:
Any one of
1. Impaired level of consciousness
2. Psychosis or delirium or confusional state
3. Grand Mal Seizure
4. Stroke or stroke syndrome
5. Aseptic meningitis
6. Mononeuritis multiplex
7. Ascending or transverse myelitis
8. Peripheral or cranial neuropathy
9. Chorea
10. Cerebellar ataxia

Category B
Any one of
1. Headache (severe unremitting)
2. Organic depressive illness
3. Chronic brain syndrome including pseudotumor cerebri
4. Disc swelling or cytoid bodies

Category C
Episodic migrainous headaches

Category D
Previous CNS disease

Category E
No previous CNS disease

CNS disease
Subsequent assessments
Category A
Acute, progressive or recurring (scored “worse” or “new”).
Any one of
1. Impaired level of consciousness
2. Psychosis or delirium or confusional state
3. Grand Mal Seizure
4. Stroke or stroke syndrome
5. Aseptic meningitis
6. Mononeuritis multiplex
7. Ascending or transverse myelitis
8. Peripheral or cranial neuropathy
9. Chorea
10. Cerebellar ataxia

Category B
Any one of the following “new” or “worse” in the last month
1. Headache (severe unremitting)
2. Organic depressive illness
3. Chronic brain syndrome including pseudotumor cerebri
4. Disc swelling or cytoid bodies
or
Any one of the following “same” or “improving” in the last month on
5. Impaired level of consciousness
6. Psychosis, delirium or confusional state
7. Grand Mal seizure

Category C
1. Episodic migrainous headaches
or
“A” 4-10 or “B” 1-4 “same” or “improving” over the last month.

Category D
Previous CNS disease

Category E
No previous CNS disease

4. MUSCULOSKELETAL DISEASE (M/S)

**Category A**
One or more of
1. Definite myositis (Bohan and Peter)
2. Severe polyarthritis with loss of function
   (not responsive to steroids < 10mg/day, antimalarials, NSAIDS)

**Category B**
One or more of
1. Arthritis
2. Tendinitis

**Category C**
1. Arthralgia
2. Myalgia
3. Tendon contractures and fixed deformity
4. Aseptic necrosis
5. Mild chronic myositis

**Category D**
Previous involvement

**Category E**
No previous involvement

5. CARDIOVASCULAR/ RESPIRATORY DISEASE (CVS/Resp)

**Category A**
Cardiac failure or symptomatic “A” effusion plus two other criteria or four from:
1. Pleuropericardial pain
2. Dyspnoea
3. Friction rub
4. Progressive CXR changes - lung fields
5. Progressive CXR changes - heart size
6. ECG evidence of pericarditis or myocarditis
7. Cardiac arrhythmias including tachycardia
   > 100 in absence of fever
8. Deteriorating lung function: > 20% of
expected or > 20% fall
9. Cytohistological evidence of inflammatory lung disease

**Category C**
Mild intermittent chest pain or one other criterion

**Category D**
Previous involvement

**Category E**
No previous involvement

**VASCUITIS**
Any one of the following

**Category A**
1. Major cutaneous vasculitis (including ulcers) accompanied by infarction occurring in the previous month
2. Major abdominal crises due to vasculitis
3. Recurrent thromboembolism (excluding strokes)

**Category B**
1. Minor cutaneous vasculitis (nail fold vasculitis, digital vasculitis, purpura, urticaria)
2. Superficial phlebitis
3. Thromboembolism (excluding strokes)- first episode

**Category C**
1. Raynaud’s phenomenon
2. Livedo reticularis

**Category D**
Previous involvement

**Category E**
No involvement
7. RENAL DISEASE
First assessment

Category A
Two or more of the following provided that 1, 4 or 5 are included
1. Proteinuria
2. Accelerated hypertension
3. Creatinine clearance < 50 ml/min
4. Active urinary sediment (on an uncentrifuged 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 (or since the previous assessment if seen less than 3 months ago)

Category B
One of the following
1. One of the category A criteria
2. Urinary dipstick 2+ or more
3. 24 hr urinary protein >0.5g but < 1g

Category C
One of the following
1. Urinary dipstick +
2. Blood pressure >140/90 (5th phase)
3. Creatinine > 130 mmol/l

Category D
Previous renal involvement

Category E
No previous renal involvement

RENEAL DISEASE
Subsequent assessments
Category A
Two or more of the following provided that 1, 4 or 5 are included

1. Proteinuria (defined as)
   (a) urinary dipstick increased by 2 or more levels or
   (b) 24 hour urinary protein rising from >0.20 g to > 1 g or
   (c) 24 hour urinary protein rising from >1g by 100% or more or
   (d) newly documented proteinuria of > 1 g

2. Accelerated hypertension

3. Deteriorating renal function (defined as)
   (a) plasma creatinine >130 μM/L or having risen to >130% of previous value or
   (b) creatinine clearance having fallen to <67% of previous value or
   (c) creatinine clearance < 50ml/min, and last time was > 50ml/min or was not measured

4. Active urinary sediment (as defined above)

5. Histological evidence of active nephritis (as defined above)

**Category B**

One of the following

1. One of the category "A" criteria

2. Moderate proteinuria (defined as)
   (a) urinary dipstick of 2+ or more or
   (b) 24 hr urinary protein rising from 1g by > 50% but <100%

3. Moderate decline in renal function (defined as)
   (a) plasma creatinine > 130μM/L or having risen to 115% of previous value

**Category C**

One of the following

1. 24hr urinary protein >0.25g

2. Urinary dipstick 1+ or more

3. Rising blood pressure (defined as)
   (a) systolic rise of ≥ 30mm Hg
   (b) diastolic rise of ≥ 15 mmHg
   (provided the recorded values are 140/90)
Category D
Previous renal disease

Category E
No previous renal disease

8. HAEMATOLOGICAL DISEASE (Hae)

Category A
One of the following
1. wcc < 1000
2. platelet count < 25
3. Haemoglobin < 8

Category B
One of the following
1. wcc < 2500
2. platelet count < 100
3. haemoglobin < 11
4. Evidence of active haemolysis (raised bilirubin +/- retic count and positive Coomb's test)

Category C
One of the following
1. wcc < 4000
2. lymphocyte count < 1500
3. platelet count < 150
4. Coomb's test positive but no evidence of active haemolysis
5. Evidence of circulating lupus anticoagulant detected by functional assays.

Category D
Previous involvement

Category E
No previous involvement