An investigation into the role of Heat Shock Proteins
in the murine lupus model, MRL/lpr

Hsp 90 in Lupus-prone mice.

Gary Bryan Faulds

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University College London, 1995

Medical Molecular Biology Unit

Department of Molecular Pathology

University College and Middlesex School of Medicine

University College London

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DECLARATION

All the work presented in this thesis is the work of Gary Faulds. Contributors by other researchers to any of the work presented is listed below.

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ABSTRACT

The role of heat shock proteins in the development of autoimmune disease remains under close scrutiny. In humans Hsp90 is overexpressed in the peripheral blood mononuclear cells (PBMCs) of approximately 30% of systemic lupus erythematosus (SLE) patients, and overexpression of this protein is associated with some types of active disease. This study was set up to investigate the role of hsps in the murine lupus-prone strain MRL/lpr. Comparable overexpression of the 90kD heat shock protein was detected in the spleens of these mice, and levels of this protein were significantly elevated compared with healthy controls ($p < 0.005$). In MRL/lpr mice elevation of Hsp90 is associated with active disease and elevated levels of Hsp90 are detected around the time of disease onset. No other tissue appeared to overexpress Hsp90, although a general rise in the levels of Hsp72, the inducible member of the 70kD heat shock protein family, was noted in a number of tissues. Neither the constitutively expressed Hsp73 protein nor the Hsp60 chaperonin were overexpressed in MRL/lpr mice compared with healthy Balb/c controls.

The immune response to Hsp90 was examined, and antibodies were found which recognised Hsp90, Hsp70 and Hsp60 in the autoimmune mice, at greater concentrations than antibodies detected in control animals. Levels of anti-Hsp90 antibodies rose with increasing age and appeared around the time of disease onset. Anti-Hsp90 antibodies were detected in approximately 60% of MRL/lpr mice over the age of 12 weeks. Levels of antibodies to Hsp90 were not correlated with other serological manifestations of the disease, however, whereas antibodies to Hsp70 and Hsp60 also rose with age and were significantly correlated in MRL/lpr mice ($p = 0.002$), and in the congenic strain MRL/++ ($p = 0.001$). Levels of these antibodies and levels of anti-Hsp90 antibodies in MRL/lpr mice were not correlated. These findings parallel results from studies undertaken on patients with SLE.

TABLE OF CONTENTS

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: HSP EXPRESSION IN MRL/*LPR* MICE

CHAPTER 4: DETECTION OF ANTI-HSP ANTIBODIES

CHAPTER 5: HSP90 PROMOTER IN CELL LINES

CHAPTER 6: DISCUSSION

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADP	Adenosine diphosphate
APS	Ammonium persulphate
ATP, CTP	Adenosine triphosphate, cytosine triphosphate
ATPase	Adenosine triphosphatase
BIC	Bicarbonate buffer
BILAG	British Isles Lupus Assessment Group
bp	Base pairs
BSA	Bovine serum albumin
°C	Degrees Celsius
C1,C2,C4	Complement (1, 2, 4)
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
Dr.	Doctor
dsDNA	Double-stranded DNA
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetra-acetic acid
eIF-2α	Eukaryotic initiation factor 2α
ELISA	Enzyme-linked immunosorbent assay
E.R.	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
g	Gram, gravity
grp	Glucose-regulated protein
HLA	Human Leukocyte Antigen

hr	Hour
HSE	Heat shock element
HSF	Heat shock factor
hsp	Heat shock protein
IDDM	Insulin-dependent diabetes mellitus
IgG, IgM	Immunoglobulin G, immunoglobulin M
Il-2, Il-6	Interleukin-2, interleukin-6
kb	Kilobase
kD	Kilodalton
M, mM	Molar, millimolar
mA	MilliAmpere
MHC	Major histocompatibility complex
mg, ml	Milligram, millilitre
min	Minute
M_r	Molecular weight
MRL/++	MRL/MpJ-+/+
MRL/*lpr*	MRL/MpJ-*lpr/lpr*
mRNA	Messenger RNA
N_2	Nitrogen
NOD	Non-obese diabetic
NZB/W	New Zealand Black/White F1 cross
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline + 0.05% Tween20
pI	Isoelectric point
PI	Propidium Iodide
PVP	Polyvinylpyrrodiline

RNA	Ribonucleic acid
RNP	Ribonucleoprotein particle
rRNA	Ribosomal RNA
r.p.m.	Revolutions per minute
RSV	Rous Sarcoma Virus
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sm	Smith antigen
SR	Steroid receptor
SSC	Standard saline citrate
ssDNA	Single-stranded DNA
SLE	Systemic lupus erythematosus
TBE	Tris-borate EDTA buffer
TBS	Tris-buffered saline
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylene diamine
TGF-β	Transforming growth factor-β
TKM	Tris, potassium and molybdate buffer
TNF-α	Tumour necrosis factor-α
TRiC	TCP-1 ring complex
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
tRNA	Transfer RNA
Tween20	Polyoxyethylene-sorbitan monolaurate
μg, μl	Microgram, microlitre
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1: INTRODUCTION

1.1: Heat shock protein biology

1.1.1. History and perspectives

In 1962 the Italian geneticist Feruccio Ritossa published a short paper reporting a hitherto unnoticed set of puffs on salivary gland polytene chromosomes of *Drosophila busckii* (Ritossa, 1962). The paper described a specific puffing pattern in these chromosomes which was induced by a temperature shock of 30°C for 30 minutes, a pattern which could be mimicked by addition of dinitrophenol or sodium salicylate. Over the course of the next decade the heat shock response (as the phenomenon was later termed) became fairly well documented at the cytological level. Following publication of a landmark set of experiments by Tissières, Mitchell and Tracy in 1974, the field of heat shock proteins (hsps) and the heat shock response really began to flourish. The authors reported synthesis of a distinct set of proteins in salivary glands, as well as in brain and Malpighian tubes, which coincided with appearance of giant chromosome puffs in *D. melanogaster* in response to heat (Tissières *et al.*, 1974). In addition to production of these proteins, it was noted that heat shock led to the disappearance or reduction of many other protein bands.

The heat shock response is now a well documented phenomenon found in all organisms, tissues and cell types, and is among the most highly conserved of genetic systems. There is much evidence to suggest that proteins produced in response to heat and other physiological stresses play a vital role in cell survival, and the remarkable degree of homology in protein sequences from bacteria to man provides a clue to the importance of the hsp families. Despite the wide variety of environments which organisms inhabit, the response to heat shock has many common features. It appears to be rapid, supporting the notion that it

is an emergency response. A number of stresses other than heat appear to universally induce the heat shock response, including ethanol, infections, anoxia and some heavy metal ions, but every organism has a characteristic spectrum of specific inducing agents which will trigger expression of heat shock proteins. Expression of these proteins appears to play a protective role, and by administration of a mild stress pre-treatment to cells, sufficient to provoke a heat shock response, increased resistance to further and more severe stress is achieved.

The response appears to be maximally induced 5-10°C above normal growth conditions for the organism, although still within the range of temperatures the organism would be expected to encounter in its natural environment. Thus in *D. melanogaster* cells, usually grown at 25°C, hsps are induced when the temperature is raised to 29-38°C (Lindquist, 1980). In contrast, fish living in near-freezing waters of the Arctic circle will produce hsps when the surrounding temperature is only 5-10°C (Lindquist and Craig, 1988), while in thermophilic bacteria, a 10°C rise above the normal growth temperature of 50°C will induce a heat shock response (Daniels *et al.*, 1984).

An interesting corollary is provided by the life cycle of some dimorphic pathogens. These organisms cycle between relatively cool temperatures in one phase and warmer temperatures encountered during another phase, their incubation in a mammalian host. The change in temperature is sufficient to trigger a strong heat shock response in the invading pathogen, and it is almost certainly no coincidence that hsps are immunodominant antigens in many of these infections (Young *et al.*, 1989).

Many of the proteins involved in the heat shock response are expressed at high levels in unstressed cells and may have important roles in normal cellular functions. Much work has gone into exploring the concept of heat shock proteins as molecular chaperones, defined by Ellis as "a family of unrelated classes of protein that mediate the assembly of other polypeptides, but are not themselves

12

components of the final functional structure" (Ellis and Van der Vies, 1991). In addition, it is known that hsps are differentially induced during embryonic development of a number of organisms (for review see Heikkila, 1993). The ubiquity and multiple functions of these proteins have interested researchers from fields as seemingly diverse as botany, genetics, molecular evolution and immunology.

1.1.2. The heat shock proteins

Heat shock proteins may be defined as those whose synthesis is sharply and dramatically induced at high temperatures (Lindquist, 1986). This interpretation excludes a related set of proteins produced during stress, collectively termed glucose-regulated proteins (grps). These proteins share sequence homology, *in vitro* ATP-binding capacity and biochemical similarity with the heat shock proteins, but are not heat-inducible and have no heat shock elements (HSEs; see section 1.1.3), thus falling outside the above definition of a heat shock protein.

There are several families of heat shock proteins, classified according to molecular weight (Table 1.1). (For a comprehensive listing of heat shock proteins see Parsell and Lindquist, 1993).

Hsp110

Hsp110, or Hsp104 in yeast, where most of the work has been undertaken, is the largest and least well-characterised of the heat shock proteins. Hsp110 is a constitutively expressed protein found in the fibrillar region of the nucleolus, the site of rRNA transcription (Ullrich *et al.*, 1986). There is some evidence to suggest that this protein may play an important role in thermotolerance (Sanchez *et al.*, 1992).

Table 1.1. The major heat shock protein families

Family	Members	Size (kD)	Functions/comments
Hsp110	Hsp110,Hsp104, *ClpA,ClpB,ClpC, ClpX*	80-110	Extreme heat tolerance ATPase activity
Hsp90	Hsp100, (gp96, grp94) Hsp90α, Hsp90β, *HtpG [C62.5]*	82-100	Cytoplasmic protein, primarily dimeric Associated with steroid receptors, protein kinases, immunophiins Possible role in protein synthesis. Weak chaperone activity
Hsp70	Several, including Hsp72 [Hsp70, Hsx70] Hsp73 [Hsc70], grp78 (BiP), grp75, *DnaK*	67-76	Bind unfolded proteins and peptides Involved in cell-cycle regulation. protein assembly, secretion, thermotolerance Associates with Hsp90 and steroid receptors Weak ATPase activity
Hsp60	Hsp60 [Hsp65, Hsp58, chaperonin 60], *GroEL* Rubisco binding protein,	58-65	Found in mitochondria Classic "molecular chaperone" Temperature-regulated ATPase activity
DnaJ	Hsp40, Hsj1, Sec63, Ydj1	40-100	Reactivation of stress-damaged substances
Small Hsps	Hsp27 [Hsp28, p29, Hsp26, Hsp23,Hsp22) Hsp18, *grpE* Histone H2B	18-47	Various functions, not really a group Hsp27 contributes to thermotolerance in mammals Hsps 27, 26, 23, 22 and 18 structurally related to α-crystallin
Hsp10	Hsp10, *GroES*	9-12	Stimulates Hsp60 functions ATP-binding ability
Ubiquitin	Ubiquitin	8	Targets abnormal proteins for degradation
Other Hsps	FKBP59 (Hsp56), Hsp47, Hsp32 (heme-oxygenase), Cyclophilin Cyp-40	Variable	Hsp56 associates with steroid receptors Peptidyl prolyl isomerase (PPI) Unknown stress functions

(bacterial homologues are shown in italics)

Hsp90

The Hsp90 gene family is highly conserved, with >40% homology between *Escherichia coli* and eukaryotic genes (Bardwell and Craig, 1987). In humans, the Hsp90 family comprises two related cytoplasmic proteins encoded by distinct genes, designated Hsp90α and Hsp90β (Simon *et al.*, 1987; Hickey *et al.*, 1989) and a protein of higher molecular weight, Hsp100 (Schlesinger, 1986). Hsp100 may be identical to grp94, a protein found in the endoplasmic reticulum that shares sequence homology with Hsp90 and is also known as gp96 (Sorger and Pelham, 1987; Srivastava, 1993). Hsp100 is induced by glucose starvation, an effect opposite to that observed with Hsp90, which is induced upon glucose restoration (Welch *et al.*, 1983; Lindquist and Craig, 1988). Hsp90 co-purifies with Hsp100 (Welch and Feramisco, 1982; Itoh *et al.*, 1990). There may be as many as 12 isoforms of Hsp90 with varying phosphorylation states (Welch *et al.*, 1983).

Complete nucleotide sequences of the Hsp90α and Hsp90β genes have been elucidated (Rebbe *et al.*, 1989; Hickey *et al.*, 1989). The transcribed region of Hsp90α contains 11 exons, ranging in size from 58 to 729 base pairs. Similarly, the Hsp90β gene comprises 12 exons, and putative heat shock elements (HSEs) are contained within the first intron of both genes. Hsp90α is less abundant constitutively than Hsp90β but more heat-inducible (Barnier *et al.*, 1987; Minami *et al.*, 1991). Hsp90α and β proteins correspond respectively to Hsp86 and Hsp84 in mice (Moore *et al.*, 1989; Moore *et al.*, 1990), and the two proteins are distinguished most notably by two amino-terminal segments in Hsp90α that are absent from Hsp90β. There is 97% homology between Hsp90β and Hsp84 (Hickey *et al.*, 1989; Lees-Miller and Anderson, 1989a), and differences in cellular specificity of expression have been reported (Gruppi and Wolgemuth, 1993). Hsp90α is isolated mainly as a homodimer, while Hsp90β exists as a homodimer and in appreciable amounts as a monomer. Higher molecular weight oligomeric forms of Hsp90β have also been reported (Minami *et al.*, 1991).

Truncation of the C-terminal of either protein leads to loss of dimerization and function (Minami *et al.*, 1994), which suggests that the functional Hsp90 protein is a homodimer. The stoichiometry of steroid receptor complexes is consistent with this notion (see below). The proteins possess ATP-binding sites and are able to autophosphorylate on serine residues (Csermely and Kahn, 1991). Hsp90α is able to be phosphorylated on two amino-terminal threonine residues by double-stranded DNA-activated protein kinase, but Hsp90β, lacking these residues, is not (Lees-Miller and Anderson, 1989b). In addition, Hsp90 is methylated in cells grown under normal conditions (Wang *et al.*, 1981) and can be ADP-ribosylated (Carlsson and Lazarides, 1983). There appears to be a site of high hydrophobicity on the surface of the molecule, and it has been suggested that this is a potential site for binding of steroid hormone receptors. The hydrophobic region may also function to act as a sensor for changes of pH, temperature and divalent cation conformation (Yamamoto *et al.*, 1991).

Hsp90 associates with a wide variety of proteins and there is a high degree of discrimination in these interactions. Hsp90 interacts with some protein kinases, including casein kinase II (Dougherty *et al.*, 1987; Lees-Miller and Anderson, 1989b; Miyata and Yahara, 1992), and eIF-2α kinase (Rose *et al.*, 1987). Particularly noteworthy is the association between Hsp90 and a number of transforming proteins with tyrosine kinase activity, including the Rous Sarcoma Virus transforming protein p60^{v-src} (Oppermann *et al.*, 1981; Brugge *et al.*, 1981; Schuh *et al.*, 1985), and tyrosine kinases coded for by the *yes, fps, fes* and *fgr* genes (Lindquist and Craig, 1988). The interaction between Hsp90 and these kinases is highly specific, and Hsp90 has been shown to modulate activity of p60^{v-src}, but not the cellular homologue p60^{c-src} *in vitro* (Xu and Lindquist, 1993). The interaction between p60^{v-src} and Hsp90 is stabilised by transition metal ions such as molybdate and vanadate (Hutchison *et al.*, 1992a).

Hsp90 is associated with a number of steroid hormone receptor complexes (Catelli *et al.*, 1985; Schuh *et al.*, 1985; Pratt *et al.*, 1992; Smith and Toft, 1992),

16

as well as tubulin (Redmond *et al.*, 1989; Fostinis *et al.*, 1992), calmodulin (Minami *et al.*, 1993), and possibly keratin cytoskeleton (Fostinis *et al.*, 1992). Furthermore, Hsp90 interacts with F-actin in a calmodulin-regulated manner (Nishida *et al.*, 1986; Koyasu *et al.*, 1986; Minami *et al.*, 1991; Miyata and Yahara, 1991), and thus may provide an anchoring mechanism for steroid receptors in the cytoplasm.

The relationship between Hsp90 and steroid hormone receptors has been intensively studied over the past decade and is probably the best characterised of the Hsp90-protein interactions. The non-transformed 8S form of a number of steroid receptors (SR) has a non-hormone-binding, non-DNA-binding component which has been characterised as a dimer of Hsp90 (Catelli *et al.*, 1985; Sanchez *et al.*, 1985; Mendel and Orti, 1988). While glucocorticoid (Housley *et al.*, 1985), progesterone (Catelli *et al.*, 1985; Hurd *et al.*, 1991) androgen (Marivoet *et al.*, 1992) and estrogen (Joab *et al.*, 1984) receptors include Hsp90 in a multi-protein complex prior to hormone binding, other members of this receptor superfamily, such as thyroid receptor and retinoic acid receptors (Dalman *et al.*, 1990; Dalman *et al.*, 1991) do not bind Hsp90. Interestingly, Hsp90 also binds to the dioxin receptor, which belongs to a class of receptors distinct from the steroid receptor superfamily (Perdew, 1988), and appears to function in an analogous manner (Pongratz *et al.*, 1992).

An early hypothesis suggested that Hsp90 acts as a cap for SR and binds to their DNA-binding sites (Baulieu, 1987). In this model, SR are in a DNA-binding conformation. Hsp90/SR complexes form immediately after receptor synthesis, and binding of Hsp90 prevents interaction of SR with DNA until incoming hormone drives dissociation of Hsp90 and SR, enabling the receptor to bind DNA. In support of this idea, Hsp90 does appear to bind to newly synthesised receptors shortly after translation (Pratt, 1993). However, reduced levels of Hsp90 have been shown to have a negative, rather than the expected positive, effect on SR action *in vivo* (Picard *et al.*, 1990) implying that Hsp90 has a

functional role in steroid hormone/receptor binding. This simple model has been superseded with the discovery of other constituents in SR complexes, namely Hsp70 (Kost *et al.*, 1989; Sanchez *et al.*, 1990b), a 55-59kD immunophilin component which may also be a heat shock protein (Sanchez, 1990; Tai *et al.*, 1992; Smith *et al.*, 1993a), and other protein components (Sanchez *et al.*, 1990a; Johnson *et al.*, 1994). The role of Hsp70 is controversial, however, and it may or may not be a genuine constituent of glucocorticoid receptor complexes, although it is clearly a component of the progesterone receptor complex (Kost *et al.*, 1989; Rehberger *et al.*, 1992; Rexin *et al.*, 1992; Hutchison *et al.*, 1992b; Alexis *et al.*, 1992). It has been argued that association of Hsp70 with unliganded glucocorticoid receptor may be a consequence of nuclear localisation in cells overexpressing these receptors (Sanchez *et al.*, 1990b; Srinivasan *et al.*, 1994), although recent work suggests Hsp70 is required to facilitate Hsp90 binding to the glucocorticoid receptor (Hutchison *et al.*, 1994).

Hsp90 attaches to the hormone-binding domain of glucocorticoid receptor, but additional sequences may be necessary for Hsp90 binding to the estrogen receptor (Chakraborti and Simons, 1991; Schlatter *et al.*, 1992). This could account for differences *in vivo*, where estrogen receptor is able to localise to the nucleus even in the absence of hormone, while hormone binding is necessary for glucocorticoid and progesterone receptors to bind DNA. Furthermore, dissociation of SR and Hsp90 is insufficient to allow SR/DNA binding and transcriptional activation (Bagchi *et al.*, 1990a; Bagchi *et al.*, 1991). A metal-linked weak leucine-zipper model has been proposed for glucocorticoid receptor/Hsp90 interaction (Schwartz *et al.*, 1993).

There is some evidence to suggest that Hsp90, Hsp70 and a third protein, p60, interact in the cell as a functional unit. Hsp90 copurifies with Hsp70 and p60, and the complex is abundant and widespread (Smith *et al.*, 1993). The heterocomplex appears to require a monovalent cation, such as K^+ or Rb^+ (Hutchison *et al.*, 1992b). It has been difficult to reversibly assemble SR/Hsp90

complexes *in vitro*, and this has only been achieved under conditions that allow Hsp70 and p60 to serve a transient role in establishing Hsp90/progesterone receptor binding in an ATP-dependent manner (Smith *et al.*, 1992b; Hu *et al.*, 1994). A similar functional unit appears to exist with p59 (Hsp56) replacing p60 (Sanchez, 1990; Sanchez *et al.*, 1990a). Data gathered to date suggest that Hsp56 and Hsp70 bind to different sites on Hsp90 but not to each other (Pratt, 1993). These complexes contain additional proteins (Perdew and Whitelaw, 1991) and have been termed *transportosomes* (Pratt, 1992). It has been suggested that transportosomes play a general role in protein folding and trafficking, and there is some evidence that these complexes may act as cytosolic molecular chaperones (Pratt, 1992; Jakob and Buchner, 1994). The functional differences between the various compositions of transportosomes have yet to be determined, although it has been proposed that the Hsp56/p60 molecule may determine direction of heterocomplex movement (Stancato *et al.*, 1993; Pratt, 1993). A "disactivation loop" model has been proposed for transportosome interactions with SR, whereby the multiprotein complex continually associates and dissociates with SR in an ATP-dependent manner (Fig.1.1.1). In a second ATP-dependent step Hsp90/SR dissociates from Hsp70 and the other protein constituents of the transportosome, allowing binding of hormone and hence receptor activation (Smith, 1993).

The disactivation model of Hsp90 and steroid hormone interaction
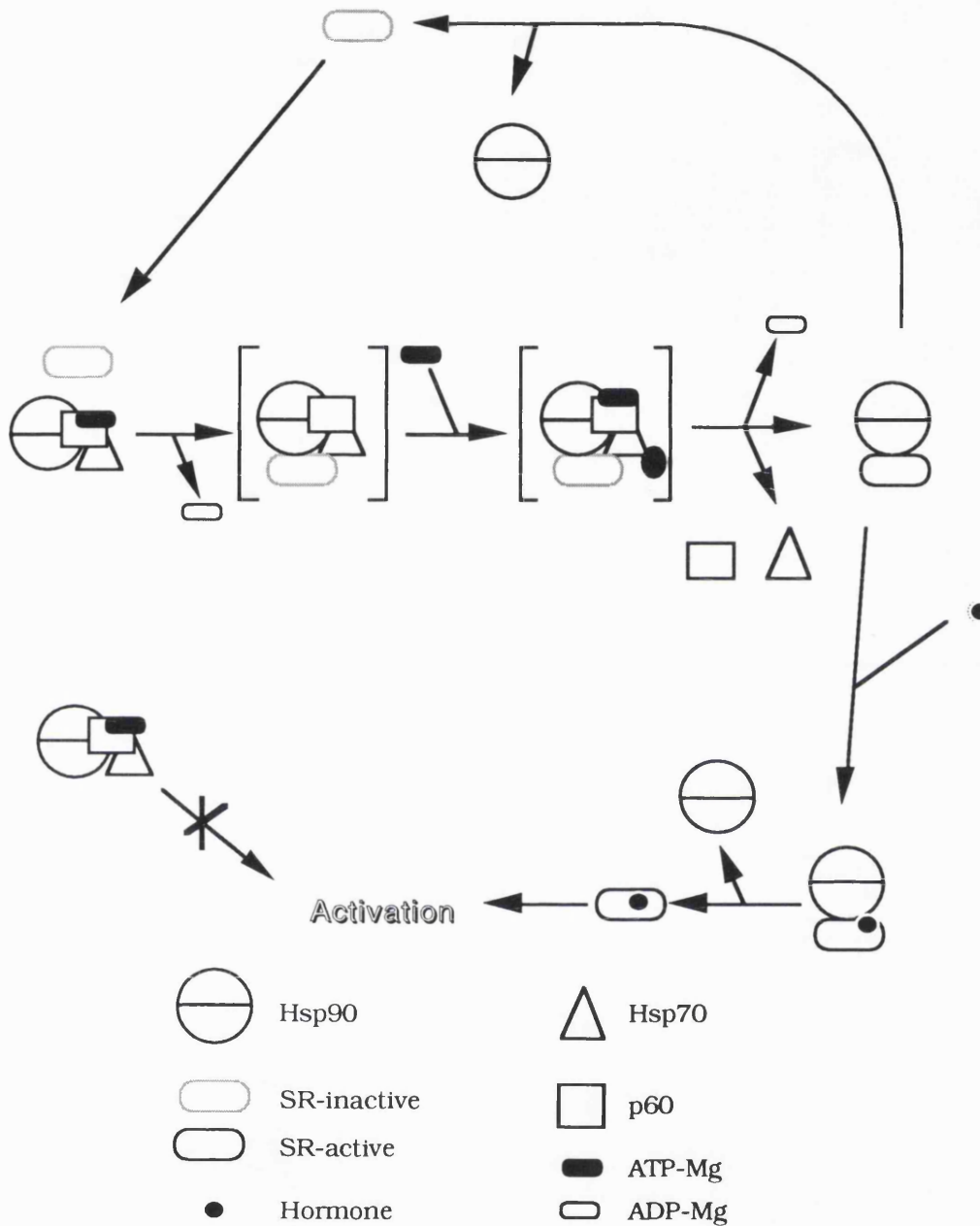
Fig.1.1.1. Steroid receptors require Hsp90 before they are able to bind hormone. In two ATP-dependent steps, receptors bind to the transportosome complex and Hsp70 and Hsp60 dissociate, allowing Hsp90/steroid receptor to bind hormone. The hormone-bound receptor dissociates in turn from Hsp90, and is now capable of binding DNA. If hormone does not bind within five minutes the receptor dissociates from Hsp90 and is unable either to bind hormone and DNA, i.e. is in a "disactivated" state. Note, however, that there may be other proteins involved in the transportosome complex. From. (Smith, 1993).

Hsp70

The Hsp70 family is a multi-gene family whose genes are expressed in a variety of physiological conditions. At least 10 Hsp70 gene-related sequences have been discovered (Mues et al., 1986), and the genes are highly conserved (Voellmy et al., 1985; Wu et al., 1985; Mues et al., 1986; Hunt and Morimoto, 1985; O'Malley et al., 1985; Gupta and Singh, 1992). Many of these genes have uninterrupted open reading frames (Hunt and Morimoto, 1985), although there are some members of the Hsp70 gene family, referred to as cognate genes, that contain introns. There is a cluster of three Hsp70 genes in close proximity to the HLA locus on chromosome 6, mapping to the major histocompatibility complex (MHC) class III region between tumor necrosis factor-α and a complement gene (Sargent et al., 1989). At least four human Hsp70-related proteins have been identified (see Morimoto and Milarski, 1990). All Hsp70 proteins have two domains, a highly conserved amino-terminal ATPase domain (Flaherty et al., 1990; Bork et al., 1992) and a carboxy-terminal portion thought to contain the peptide-binding sites. The ATPase domain shares structural similarities with actin and hexokinase ATPase domains (Bork et al., 1992). The major heat-inducible Hsp70 protein, Hsp72 (also known as Hsp70, Hsx70, Hsp68, 72K) is the major translation product of heat-shocked cells. Hsp72 has a low basal expression and is under control of the adenovirus EIA protein (Nevins, 1982). Hsp72 may be important in development, as protein expression is also cell-cycle regulated (Milarski and Morimoto, 1986). It appears to be limited to primate cells, having no counterpart in rodents. Hsp73 (Hsc70, Hsc73, 73K, clathrin uncoating ATPase) has a high constitutive level of expression and is only slightly heat-inducible. There is some confusion in the literature, but there appears to be a third member of the Hsp70 family which is not expressed constitutively and appears only on heat shock. Alternatively, this may be an isoform of Hsp72, as several isoforms are known to exist, their expression dependent on the preceding stress and cell type investigated (Welch et al., 1983). Another member of the

family, grp78 (BiP), is found in the endoplasmic reticulum, has a high constitutive level of expression which is enhanced by glucose deprivation, and is not induced upon heat shock. This protein thus falls into the definition of a glucose-regulated protein, as does another member of this family, grp75 (mtp70), found in mitochondria. Hsp72 and Hsp73 are 85% identical at the amino acid level, while Hsp72 and grp78 are 76% identical (Hunt and Morimoto, 1985). Development of thermotolerance has been linked with expression of these proteins (Lindquist and Craig, 1988; Angelidis *et al.*, 1991; Li *et al.*, 1992).

Hsp70 proteins interact with nascent polypeptide chains and maintain them in loosely folded conformations (for reviews, see Hartl and Martin, 1992; Gething and Sambrook, 1992; Becker and Craig, 1994). Hsp70 can recognise and bind unfolded proteins *in vitro*, releasing the proteins in an ATP-dependent manner (Beckmann *et al.*, 1990; Palleros *et al.*, 1991; Beckmann *et al.*, 1992). There is a requirement for ATP-binding and K^+ in Hsp70/protein dissociation, but ATP hydrolysis is unnecessary (Palleros *et al.*, 1993). Protein release can be inhibited by the addition of ADP (Palleros *et al.*, 1991). It has been proposed that Hsp70 slows down folding of nascent proteins until transcription is complete, thereby functioning as an *unfoldase* (Beckmann *et al.*, 1990; Ellis and Van der Vies, 1991). Grp75 in mitochondria may play an analogous role, facilitating unfolding of the polypeptide chain for preprotein transport across the outer membrane (Kang *et al.*, 1990; Gambill *et al.*, 1993). A second function for grp75 has been suggested, implicating this protein in translocation of preproteins into the mitochondrial matrix (Voos *et al.*, 1993). Similarly, Hsp70 is required for importing proteins into perioxosomes (Walton *et al.*, 1994).

The function of Hsp70 in steroid hormone signalling remains unclear, although there is a suggestion that Hsp70 may influence DNA-binding and/or transcriptional activities of glucocorticoid receptors (Srinivasan *et al.*, 1994).

The role of Hsp70 and Hsp60 in protein folding
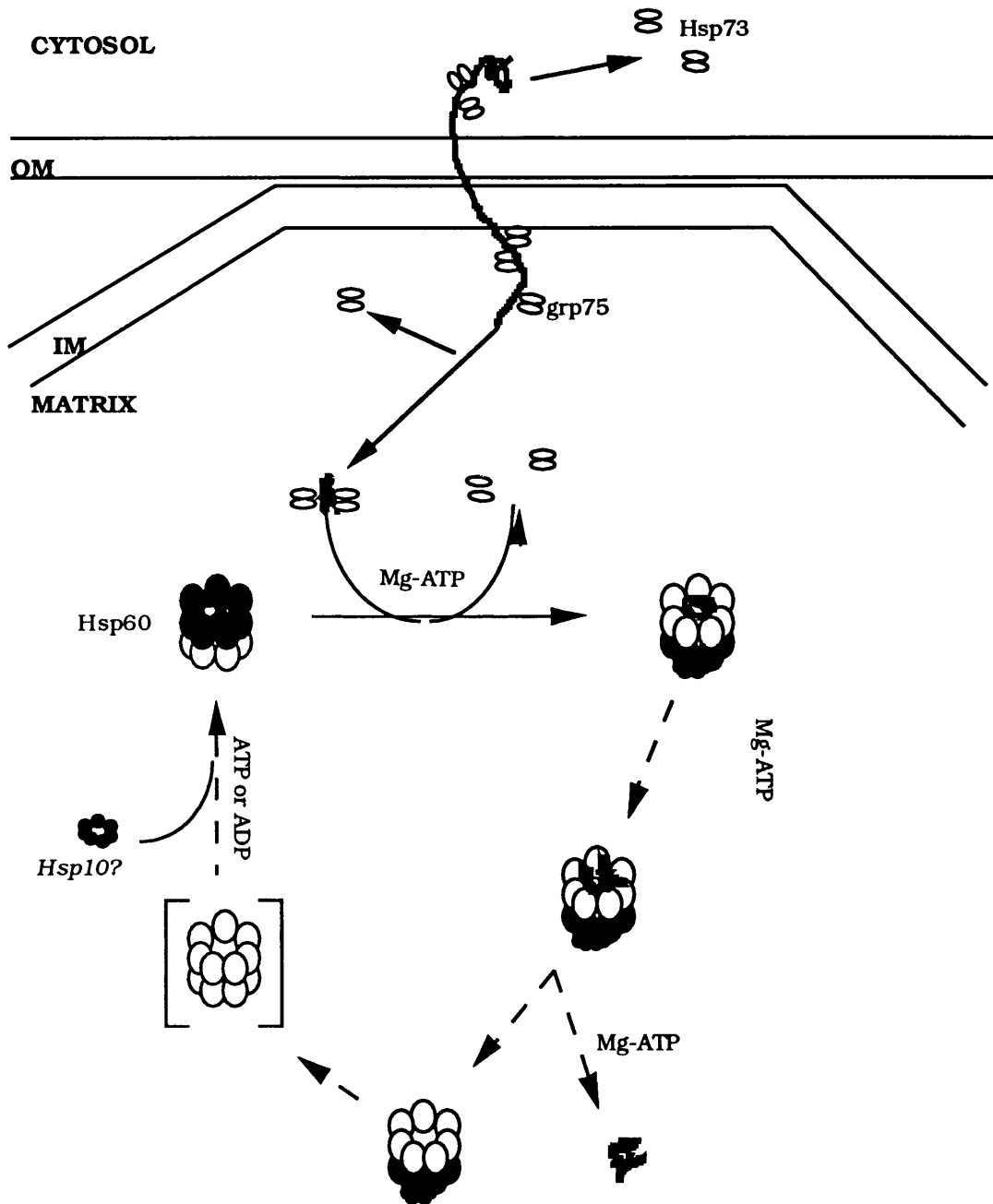
Fig. 1.1.2. Hsp70 and Hsp60 may act sequentially to facilitate correct protein folding in mitochondria. Hsp73 is involved in the translocation of nascent polypeptides across the mitochondrial membrane. Following translocation grp78 transfers the polypeptide chains to the Hsp60 oligomer, which subsequently chaperones correct folding. Hsp10 also appears to be involved in this cycle, possibly functioning as an ATP donor for Hsp60. Adapted from (Hartl and Martin, 1992). Reviewed by (Manning-Krieg et al., 1991; Gething and Sambrook, 1992; Hendrik and Hartl, 1993).

Alternatively, it has been proposed that Hsp70 effects ATP-dependent unfolding

of SR, thus allowing recognition and binding by Hsp90 (Hutchison *et al.*, 1994;

Jakob and Buchner, 1994).

Hsp60

Hsp60 proteins are found in the cytosol of bacteria, where they are known as

GroEL proteins, in the matrix of mitochondria, and in the stromal compartment

of chloroplasts. Most of these proteins share a common oligomeric structure, a

so-called "double doughnut" of two seven-membered heptameric rings (for

review, see Ellis and Van der Vies, 1991), although a single heptameric ring with

functional activity has been observed in mammalian mitochondria (Viitanen *et*

al., 1992). Each Hsp60 molecule is capable of binding one ATP molecule, thus

allowing for 14 ATP-binding sites on the oligomeric complex (Bochkareva *et al.*,

1992). The proteins are highly conserved, most sharing greater than 60%

homology (Hemmingsen *et al.*, 1988; Reading *et al.*, 1989).

The primary function of Hsp60 appears to be that of a molecular chaperone,

facilitating correct folding of polypeptides. With regard to this function it has

been proposed that Hsp70 and Hsp60 act sequentially in a common pathway

(Fig.1.1.2). However, it is important to note that while the yield of correctly

folded polypeptides is enhanced through Hsp60 mediation, the actual rate of

folding appears to be somewhat decreased (Parsell and Lindquist, 1993). Hsp60

binds to a multitude of unfolded proteins, but not in their folded configurations

(Bochkareva *et al.*, 1988; Lecker *et al.*, 1989).

The most likely site of interaction between Hsp60 and unfolded proteins appears

to be the central cavity of the oligomer (Langer *et al.*, 1992; Georgopoulos and

Welch, 1993). The cavity is seemingly large enough to contain a protein of 75kD

(Langer *et al.*, 1992). Oligomeric Hsp60 appears to function in co-operation with

a similar seven-membered ring structure of a co-chaperone, Hsp10 (Hartman *et*

al., 1992). Binding of ATP and the Hsp10 ring is necessary for release of the

majority of folded proteins from the Hsp60 complex. The role of Hsp10 is not fully understood, but the bacterial homologue GroES binds ATP co-operatively with an affinity similar to that of GroEL, despite lacking detectable ATPase activity, and a role as an ATP donor has been proposed (Martin *et al.*,1993). Hsp60 has weak ATPase activity that increases with temperature (see Parsell and Lindquist, 1993), and binding of Mg-ATP induces a conformational change in the heptameric ring, leading to the release of folded proteins (Ostermann *et al.*, 1989).

Based on experimental observations, the following model of the role of GroEL (and by analogy Hsp60 in mitochondria) in protein folding has been suggested (Hendrick and Hartl, 1993):

A. The single-ring model

> (1) Initial interaction between GroEL and protein is restricted to one of the heptamer rings, probably the ring distal to that bound to GroES.

> (2) Co-operative ATP hydrolysis, enhanced by GroES, is accompanied by a concerted release of the bound protein, thus making a number of folding elements available.

> (3) Folding then proceeds, but immediate exit of the released protein from the central cavity may be retarded by GroES-induced conformational changes in GroEL. A partially folded protein may re-bind and undergo another cycle.

> (4) The bound protein is released in folded conformation.

B. The double ring model

Taking into account experimental data showing functional activity of a single Hsp60 ring (Viitanen *et al.*, 1992) the above model can be postulated. However, an alternative model, based on the full 14-membered oligomer, has also been proposed (Creighton, 1991):

(1) If via ATP hydrolysis one ring has converted to a state with low affinity for substrate the other will preferentially adopt a high-affinity conformation.

(2) An unfolded protein shuttles between the two rings, thus advancing along the folding pathway until all the binding sites are hidden in the interior of the folded molecule. The protein will only migrate from the first to the second rings if further folding is necessary.

(3) Fully folded protein is released on hydrolysis of Mg-ATP.

Hsp60 is induced by heat two- to three-fold (McMullin and Hallberg, 1987), in contrast to GroEL, which strongly increases expression with increased temperature and may account for 10-15% of total cellular protein in *E.coli* (Neidhardt *et al.*, 1984). Hsp60 associates with a wide variety of proteins at high temperatures and also prevents aggregation of proteins that denature at physiologically relevant temperatures *in vitro* (Martin *et al.*, 1992).

TRiC

The notion that proteins in bacteria and the mitochondrial matrix require molecular chaperones has led to a search for an equivalent molecule in the eukaryotic cytosol. The best candidate thus far is probably a hetero-oligomeric structure termed TRiC, with a 60kD protein TCP-1 (T-complex polypeptide 1) as the predominant molecule (Gao *et al.*, 1992; Parsell and Lindquist, 1993; Hendrick and Hartl, 1993). The TRiC proteins share low but significant levels of amino acid identity with the Hsp60 family (Lewis *et al.*, 1992), and TCP-1 shares 30-40% identity with a nine-membered ring structure termed TF55 (for thermophilic factor 55) isolated from thermophilic archaebacteria (Trent *et al.*, 1991). However, these proteins are not heat-inducible (Parsell and Lindquist, 1993), and TRiC is unable to chaperone the folding of some proteins (Gao *et al.*, 1993). The role of TRiC in protein folding thus demands further investigation before it can genuinely be regarded as the cytosolic equivalent of Hsp60.

Small Hsps

The small hsps are the least conserved of the heat shock protein families, and vary greatly in size and protein sequence. They constitute a distinct family on the basis of 15-20% amino acid identity (including a conserved hydrophobic C-terminal sequence) and similar hydropathy profiles (Parsell *et al.*, 1993). In *Drosophila* there are four related proteins, Hsp27, Hsp26, Hsp23 and Hsp22, but vertebrates and yeast appear to express only one 27/28kD protein, usually referred to as Hsp27 in humans and Hsp25 in rodents. However, there is significant homology to the α-crystallin lens protein, and there appears to be a cluster of three Hsp27 genes and one pseudogene in humans (Hickey *et al.*, 1986a; Hickey *et al.*, 1986b). Only one gene, sharing a similar arrangement of functional motifs, has been found in mice (Gaestel *et al.*, 1993), although a pseudogene sharing 99% amino acid identity with Hsp25 has recently been discovered (Fröhli *et al.*, 1993). The protein can be phosphorylated, and exists in at least three isoforms (Arrigo and Welch, 1987). Secondary structure of all small hsps tends to dominated by β-sheet formation (Jakob and Buchner, 1994).

Hsp27 is localised to eukaryotic cytosol, normally within the perinuclear region in close proximity to the Golgi, translocating to the nucleus upon heat shock (Arrigo *et al.*, 1988). Hsp27 is heat-inducible and has an oligomeric structure with a native molecular weight of at least 500kD that sediments on sucrose gradients at 15-20S. (Arrigo and Welch, 1987; Parsell and Lindquist, 1993). These proteins display an ATP-independent ability to act as molecular chaperones *in vitro* (Jakob *et al.*, 1993), and may have a role in thermal resistance, with different isoforms dominant depending on the physiological state of the cell (Arrigo *et al.*, 1988; Rollet *et al.*, 1992). Hsp27 associates with actin, and changes in the phosphorylation state of Hsp27 have been shown to result in modulation of actin filament dynamics and fluid phase pinocytosis (Lavoie *et al.*, 1993), suggesting an essential role in signal transduction.

Ubiquitin

Ubiquitin is a protein of 76 amino acids found in all eukaryotic cells. It is highly conserved, with only three amino acids differing in the yeast and human proteins. It is synthesised as a polyprotein, generally consisting of tandem repeats of the protein coding sequences (Lindquist and Craig, 1988). Four amino acids - leu-arg-gly-gly- extend outward from the carboxy-terminal of the protein and the second glycine residue can form an isopeptide bond to an ε-amino group of a lysine in a target protein (Chau et al., 1989). Ubiquitin is capable of recognising proteins with disturbed spatial structure and binding them, becoming a target for cellular proteinases (Schlesinger, 1986). Conjugation of ubiquitin to proteins destined for degradation generally proceeds through three steps (reviewed in Ciechanover, 1994):

(i) Activation of ubiquitin C-terminal glycine by ATP, catalysed by ubiquitin-activating enzyme E1.

(ii) Transfer of ubiquitin by ubiquitin carrier protein E2 from E1 to a substrate bound to a ubiquitin-protein ligase, E3.

(iii) Polyubiquitination by an E3-dependent mechanism, successive ubiquitins conjugated through Lys-48 to the preceding protein.

Following marking of a protein to ubiquitin the conjugated protein is selectively degraded in an ATP-dependent process by a subunit of a 26S protease complex, recycling free ubiquitin (Deveraux et al., 1994). The recycling process is carried out by a ubiquitin C-terminal hydrolase (isopeptidase).

Ubiquitin-conjugation is required for many functions in the cell aside from marking proteins for degradation, including DNA repair and replication and rRNA processing (Schlesinger, 1990b; Hershko and Ciechanover, 1992). For example, ubiquitin may have a role in regulatory pathways. Several cell surface receptors, including lymphocyte homing receptor and growth hormone receptor, can be modified by ubiquitin, although the function of this modification remains obscure. The ξ- and CD3 δ- subunits of the T cell receptor can also be

ubiquitinated, and this occurs in response to receptor engagement (Cenciarelli *et al.*, 1992; Ciechanover, 1994). The ubiquitin pathway is also utilised in activation of transcription factor NF-κB (Palombella *et al.*, 1994). A pivotal role for this heat shock protein in antigen processing has been demonstrated with the discovery that generation of peptides presented on MHC class I molecules can be blocked by inhibition of the proteasome (Rock *et al.*, 1994).

Other Hsps

Several other proteins appear to be heat-inducible, including members of two functionally related but structurally distinct families whose functions have yet to be fully understood, the cyclophilins and the FK506-binding protein (FKBP) family. Both these protein families are peptidyl prolyl *cis-trans* isomerases, i.e. they catalyse Xaa-Pro isomerization, and as such have a direct role in protein folding (Parsell and Lindquist, 1993). However, not all of these proteins are heat-inducible, although it is notable that FKBP52 (Hsp56), found in the transportosome complex with Hsp90 and Hsp70, does have increased expression following heat stress. Similarly, the cyclophilin CyP-40, which has a carboxy-terminal domain with structural similarities to Hsp56, is also found as a component of inactive steroid receptor complexes (Fruman *et al.*, 1994). Other hsps include Hsp47, a collagen-binding membrane protein found in murine and avian fibroblast cells, and Hsp32, which is identical to heme-oxygenase in rats and is present in microsomes. Interestingly, the Hsp47 gene, consisting of six exons separated by five introns, has an alternatively spliced mRNA that is expressed during heat shock, and may prove a useful *in vivo* model for studying heat shock-induced expression (Takechi *et al.*, 1994).

1.1.3. The heat shock response

The nature of the response

In response to an increase in temperature cells and organisms switch their transcriptional machinery to synthesis of heat shock protein RNAs, at the same time repressing transcription of previously active genes and translation of pre-existing messages (for review see Lindquist, 1986). Two classes of polysomes are seen, an active class in which high levels of stress-induced mRNAs are being translated and an inactive class containing mRNAs that were active prior to the cellular stress. Prior induction of heat shock proteins appears to confer thermotolerance on at least some cells, although there is still considerable debate as to whether induction of hsps is sufficient for acquisition of thermotolerance (Alexandrov, 1994). The heat shock response is rapid, and in *Drosophila* new polytene chromosome puffs can be seen within one minute of applying the stress, reaching a peak between 30 and 60 minutes.

Upon heat shock, cells undergo a number of morphological changes. Mitochondria appear swollen, with more prominent cristae and larger intercristal spaces, and more mitochondria are observed near and around the nucleus. The Golgi apparatus appears to fragment somewhat, with an increase in number of vesicularized membranes found throughout the nuclear region. Vimentin-containing intermediate filaments in the cytoplasm collapse and aggregate in and around the nucleus, but no similar changes are seen in microtubules. There is an increase in number of actin-containing fibres that are found spanning the cytoplasm of stressed cells, and a number of unusual rod-like structures can be seen within cell nuclei, shown upon investigation to be actin-containing inclusion bodies. A dramatic loosening of the nucleolus is evident in stressed cells, and there is a loss in the amount of granular components after heat shock treatment (Welch and Suhan, 1985). These changes are reversible, and cells

gradually regain their former morphology upon restoration of normal temperature.

Regulation of the heat shock response

There appears to be a common mechanism involved in transcriptional regulation of all eukaryotic heat shock genes. All heat shock genes appear to be found in an open configuration at normal temperatures and all organisms have at least one copy of a rotationally symmetric consensus element, now referred to as a heat shock element (HSE), in the 5' region of these genes (Pelham, 1982). HSEs have variable numbers of the consensus sequence nGAAn, arranged in alternating orientation, and at least two nGAAn units are required for high affinity binding *in vitro* (Xiao and Lis, 1988; Perisic *et al.*, 1989). HSEs do not function as basal promoter elements, although interactions with basal elements are required for maximum transcription under stress (Williams and Morimoto, 1990). There are other unusual features of heat shock gene promoters, including an RNA polymerase II molecule which is bound and paused after transcribing only a small percentage of the gene (O'Brien and Lis, 1991). Pausing continues even after heat induction (Giardina *et al.*, 1992) and studies with hybrid promoters in *Drosophila* have shown that upstream sequences of these genes appear to be involved in pausing the polymerase (Lee *et al.*, 1992).

A heat shock transcription factor (HSF) which binds HSEs has been found and characterised (Parker and Topol, 1984; Sorger, 1991). In unstressed human and *Drosophila* cells HSF is present in both the cytoplasm and the nucleus as a monomer that does not bind DNA, and in response to stress HSF forms trimers and accumulates in the nucleus. HSF binding to *Drosophila* Hsp70 promoter HSEs is co-operative, indicating a need for stereoalignment of these elements (Amin *et al.*, 1994), and HSF may require Ca^{2+} for activation (Price and Calderwood, 1991).

31

HSF/DNA binding activity does not always correlate with transcriptional activation. Yeast HSF, for example, is bound to DNA as a trimer before and after heat shock (Sorger and Nelson, 1989), and there may therefore be other steps involved in the activation pathway. There is a hydrophobic heptad repeat in the heat-shock inducible HSFs that is absent from the yeast HSF which may account for the former's suppression of trimerization (Lis and Wu, 1993). Human HSFs are unable to bind *in vitro* to nucleosome-bound HSEs (Taylor *et al.*, 1991) and it has recently been shown that the GAGA transcription factor is able to disrupt chromatin structure, so this factor may have a crucial role in setting up heat shock genes for effective transcription (Tsukiyama *et al.*,1994). In addition, HSFs are able to be phosphorylated, and this may affect the protein's ability to modulate transcriptional activation (Hunt and Morimoto, 1985).

It has been proposed that heat shock proteins may negatively regulate heat shock gene expression via an autoregulatory loop, such that increased numbers of misfolded proteins induced during heat shock appropriate Hsp70, which in turn activates HSF. Hsp70 may thus have an important role to play in regulation of HSF activation, and Hsp70 has been shown to block *in vitro* activation of HSF from a non-DNA-binding to a DNA binding state (Abravaya *et al.*, 1992). Furthermore, complexes containing Hsp70 and trimeric HSF have been observed in heat-shocked cells (Abravaya *et al.*, 1992), and the heat shock transcriptional response is correlated with levels of denatured and misfolded proteins (Morimoto *et al.*, 1992).

Morimoto has proposed the following model for HSF activation (Morimoto, 1993):

> (i) Under non-stressful conditions HSF is maintained in non-DNA binding form. Hsp70 may associate with HSF as it does with nascent proteins and stabilise this conformation.

(ii) Upon heat shock, the increased pool of misfolded proteins competes with HSF for Hsp70 association. Heat shock thus acts to overcome negative regulation of HSF by Hsp70.

(iii) The released HSFs form trimers, bind DNA and activate hsp transcription. Activation of HSF in higher eukaryotes is ATP-regulated, and appropriate phosphorylation of HSF is also required for transcriptional activation. Increased expression of Hsp70 then acts as a regulator, as Hsp70 will reassociate with HSF and inhibit further hsp transcription.

This simple picture has been complicated somewhat by the discovery of a family of HSFs containing at least three members, HSFs 1, 2 and 3. These proteins share several structural features, including an amino-terminal DNA binding domain, a cluster of hydrophobic amino acids arranged into leucine zippers adjacent to that domain, and a carboxy-terminal leucine zipper. The DNA binding domain is a unique variant of a helix-turn-helix motif (Harrison *et al.*, 1994).

Also unique among DNA binding proteins, all three HSFs form trimers; these appear to be homotrimers as there is no evidence for formation of mixed oligomers (Rabindran *et al.*, 1993). The three HSFs are expressed in most, but not all cells, and there are functional differences among the three which may account in part for the differential nature of the heat shock response to different stressors. In this regard it is interesting to note that HSF1 mediates the response to heat shock and displays stress-induced DNA-binding ability, oligomerization and nuclear localisation, while HSF2 is activated by hemin during erythroid development, and does not respond to heat stress (Sistonen *et al.*, 1994; Morimoto, 1993; Sarge *et al.*, 1993; Kroeger *et al.*, 1993). Hemin treatment followed by heat shock results in synergistic induction of Hsp70 gene transcription (Sistonen *et al.*, 1994). HSF3 is activated under conditions different to those which activate either HSF1 or HSF2 (Nakai and Morimoto, 1993).

RNA metabolism

In a classical heat shock response cells switch their transcriptional machinery to the synthesis of hsps, concurrently down-regulating normal protein synthesis. Heat shock affects other aspects of RNA metabolism, including mRNA degradation and RNA splicing. A block in effective RNA splicing observed at high temperatures may in part explain why intronless genes like inducible Hsp72 are able to be processed under heat shock conditions while intron-containing genes are not. (Yost *et al.*, 1990). However, some heat shock genes do contain introns and are still up-regulated during stress, e.g. Hsp90; presumably splicing of these RNAs is somehow more heat-resistant than other non-heat-shockable RNA transcripts. Selective deadenylation of Hsp70 transcripts may also play a role in regulation of Hsp70 expression, as there is evidence to suggest that adenylated Hsp70 mRNAs are more efficiently translated than deadenylated mRNAs, and deadenylation of Hsp70 mRNA poly (A) tails decreases dramatically on heat shock. Hsp90 mRNA transcripts degrade more slowly than Hsp70 transcripts, correlating with adenylation state of the respective mRNAs (Dellavalle *et al.*, 1994).

1.1.4. Heat shock proteins and development

Heat shock proteins appear to play specific roles in the development of virtually all organisms. In mammals, embryos lack the ability to undergo a heat shock response until the blastocyst stage and expression of at least some heat shock genes appears to be developmentally regulated and independent of the presence of HSEs. Whole rat embryos exposed to a 42°C heat shock show cell cycle arrest at both G1/S and S/G2 boundaries followed by a slow progression through S phase, resulting in a 2-3hr lengthening of cell cycle time, and cell recovery appears to be associated with Hsp72 expression.

Other hsps, including Hsp90, Hsp73 and Hsp27, are induced at specific cell cycle phases (Walsh and Li, 1993). Hsp90 mRNA, for example, is maximally expressed during the G1/S phase transition, although its subcellular localisation remains constant, including its association with microtubules (Redmond *et al.*, 1989; Walsh *et al.*, 1993). This contrasts with Hsp73, which is also overexpressed at the G1/S transition phase, but is localised to the nucleus in a cell-cycle dependent manner (Jérôme *et al.*, 1993). A recent paper has provided evidence that Hsp90 may also induce condensation of the chromatin structure (Csermely *et al.*, 1994).

In the normal mouse thymus, immature double negative (CD4$^-$CD8$^-$) T cells are better able to survive heat shock than mature single positive (CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$) or double positive (CD4$^+$CD8$^+$) T cells. Furthermore, thymocytes from adult mice terminate the heat shock response much more quickly than embryonic thymocytes or mature spleen T cells (Mosser *et al.*, 1993).

The picture emerging from the study of heat shock proteins and the heat shock response is that of related families of proteins which have evolved to facilitate a variety of normal cellular functions, including correct and efficient transport and folding of nascent polypeptides, 'priming' of steroid hormone and other receptors, and recognition of misfolded or damaged proteins. In response to potentially damaging stresses these proteins act to shield cells from the worst excesses of the stress, and ensure that cells can regain normal functions as quickly as possible once the stress has been alleviated. The association of hsps with so many proteins of potential medical interest has led to an investigation of the role of these proteins in disease pathogenesis, which will be reviewed in the following section.

1.2: Heat Shock Proteins and Disease

The heat shock response appears to be primarily protective, providing cells with a mechanism for dealing with stress, in effect "buying time" for cell survival. Through shielding proteins that have already been translated and keeping them in a state that enables them to take up functional conformations rapidly once the stress has been alleviated, the chaperoning role of hsps fits in well with this notion. However, the ubiquitous expression of hsps throughout phylogeny, their highly conserved sequences and the discovery that they are immunodominant antigens in infection (see below) has led to investigations into the role of these proteins in infectious and autoimmune diseases, and more recently cancer.

1.2.1. Hsps and the immune response

A link between heat shock proteins and the immune response has been investigated, and evidence has been found to suggest these proteins may have functions within this context. Lymphocyte and macrophage activation by interleukins and mitogens induces increased levels of hsps, including Hsp90 and Hsp73 (Granelli-Piperno et al., 1986; Haire et al., 1988; Ferris et al., 1988). Human interleukin-2 (Il-2) dependent T cells, for example, increase synthesis of Hsp73 15-fold following treatment with Il-2, while Hsp90 is rapidly and transiently increased (Ferris et al., 1988). Mitogen-activated hsp synthesis in lymphocytes is diminished in aged donors, paralleling a decrease in proliferative response (Faassen et al., 1989). An Hsp70 gene has been mapped within the human MHC complex, located between the loci for complement genes and tumour necrosis factor (Sargent et al., 1989). Heat shock renders target cells resistant to natural killer cells and lymphokine-activated killer cells (Kaufmann, 1990).

36

The formation, expression and secretion of immunoglobulin molecules requires grp78 (BiP) as a molecular chaperone, and a peptide binding protein with a role in antigen presentation shares many features with Hsp70, and may be a member of this heat shock protein family (Vanbuskirk *et al.*, 1989). A multitude of potentially harmful immune mechanisms, such as cytotoxic CD4$^+$ and CD8$^+$ T cells, $\gamma\delta$ T cells, natural killer cells and cytokines, have been shown to be activated by bacterial Hsp60, leading to the concept of molecular mimicry and potential autoimmune disease (reviewed in Kiessling *et al.*, 1991). Ubiquitin has been implicated in MHC class I-restricted antigen presentation through the ubiquitin-dependent proteolytic pathway (Michalek *et al.*, 1993). It has been suggested that grp94 (gp96) may function as a peptide acceptor to facilitate MHC class I-peptide assembly in the lumen of the endoplasmic reticulum (Li and Srivastava, 1993), and other immune system functions appear to be mediated by stress proteins, including lymphocyte homing, resistance to target cell lysis, tumor antigenicity and immune surveillance (for review, see Winfield and Jarjour, 1991).

One question that needs to be addressed is how molecules such as hsps, normally found intracellularly, become subject to immune system recognition. Despite a lack of appropriate signal sequences, however, there is evidence to suggest that these molecules are capable of being expressed on cell surfaces (Jarjour *et al.*, 1990). Four possible mechanisms have been suggested whereby hsps might be expressed on the surfaces of cells (Winfield, 1989):

(1) Processing and presentation in the context of MHC molecules of a cell's own intracellular self hsps or hsps released by other cells in a manner similar to that of non-self proteins generally.

(2) Translocation of hsps to the cell surface as they chaperone MHC or other nascent integral membrane proteins to the plasma membrane.

(3) Direct binding of self hsps released from cells to MHC molecules or other cell surface proteins.

(4) Expression of hsps or closely related proteins on the plasma membrane as integral membrane proteins.

Hsps and T cells

Hsp-specific antibodies and T cells have frequently been identified (Munk *et al.*, 1989; Kaufmann, 1990), and there is evidence to suggest that (i) self-hsp reactive T cells are not completely eliminated by thymic deletion, and (ii) these T cells are an integral part of the immune network (Munk *et al.*, 1989; Kaufmann, 1994). A subset of T cells bearing a novel T cell receptor heterodimer, the γδ receptor, has been identified, and it has been suggested that γδ T cells may function through a mechanism different from the better understood αβ T cells, perhaps through reactivity with molecules like hsps, allowing for postulated functions like immune surveillance of epithelia and macrophage activation (Born *et al.*, 1990b). It appears likely that γδ T cells recognise stressed cells, as experiments with murine CD8$^+$ T cells specific for a bacterial hsp showed that self-hsp reactive T cells are capable of recognising stressed cells while leaving unstressed cells untouched (Koga *et al.*, 1989). Furthermore, when T cells are incubated at temperatures sufficient to induce a heat shock response there is selective proliferation of γδ T cells, and a similar phenomenon is seen *in vivo* on pre-exposure to mycobacterial antigens (Rajasekar *et al.*, 1990). T cells bearing the γδ T cell receptor have been found in experimental arterial injuries, localised to sites expressing Hsp60 (Heng and Heng, 1994) and a link between Hsp70 expression and distribution and γδ T cell distribution and maturation has been noted (Bell, 1993). In addition, human γδ T cells are capable of responding to the mycobacterial Hsp60 homologue, Hsp65 (Haregewoin *et al.*, 1989) and neonatal thymus γδ-receptor-expressing cells which recognise Hsp65 are self-reactive (O'Brien *et al.*, 1989). This self-reactivity may involve surface expression of Hsp60 peptides (Born *et al.*, 1990a), and a protein recognised by antibodies to rabbit Hsp60 is constitutively expressed on surfaces of human γδ T cells, but is

not found on T cells expressing αβ receptors (Jarjour *et al.*, 1990). The relationships between hsps, γδ T cells and disease will be considered in more detail below.

1.2.2. Hsps and Infectious diseases

Many dimorphic pathogens have to face a sudden rise in temperature on transmission from vector to host, sufficient to trigger a heat shock response. Interestingly, the host response also involves induction of hsps, which may be due to the host's need to protect itself from noxious molecules produced by phagocytes (Kaufmann, 1990). Hsp70 gene products are major mycobacterial antigens, and antibodies to a 75kD antigen, closely related to Hsp70, have been isolated from patients infected with the malarial parasite *Plasmodium falciparum*. (Kumar *et al.*, 1990). Numerous other examples of heat shock proteins, particularly Hsp70 and Hsp60, as antigens in other parasitic and bacterial infections have been documented (reviewed in Young *et al.*, 1989; Young and Elliott, 1989).

In patients with systemic candidiasis a 47kD breakdown product of *Candida albicans* Hsp90 is an immunodominant antigen. It is found in large amounts in sera of infected patients, as well as in their urine and in circulating immune complexes, and antigen titre correlates with clinical condition, increasing with deterioration in patients' condition. Immunisation with antibodies to a conserved epitope of Hsp90, LKVIRK, halved mortality in animal studies (Matthews *et al.*, 1991), and a good antibody response in patients is associated with recovery, observations which imply that circulating fungal Hsp90 in an extracellular environment is harmful. Experiments in *Saccharomyces cerevisiae* have shown that mammalian Hsp90 can render viable yeast that have had their Hsp90 genes removed (Picard *et al.*, 1990), suggesting considerable functional cross-reactivity between yeast and mammalian heat shock proteins. Sera from patients with a

variety of diseases have been epitope mapped to the human Hsp90 protein sequence, and two epitopes, with sequences NNLGTI and KILKVIRK, were recognised by 7/9 patients with systemic candidiasis, although patients with other fungal diseases recognised these epitopes less frequently (Al-Dughaym *et al*., 1994). Epitope mapping of an Hsp70 antigen from *M. leprae* revealed two peptide sequences in the molecule which were recognised by PBMC from a human donor, and one of these sequences was homologous to the human protein, implying that this epitope had the potential to stimulate an autoimmune response (Adams *et al*., 1993).

1.2.3. Hsps and viral infections

The deciphering of the vital roles hsps play in protein folding in cells and the discovery that aberrant polypeptides are sufficient to generate an hsp response have provided a reason for the host stress response induced during virus infections. It is conceivable that viruses, lacking their own hsps, utilise this response by hijacking the host's cellular machinery to ensure efficient translation of viral proteins. Any T cell response generated to hsps during a viral infection must therefore be directed against a host protein expressed ubiquitously at high levels. However, there is some selectivity regarding the induction of hsps. For example, adenovirus, vaccinia and herpes virus induce Hsp70 gene transcription in human and monkey cell lines, but SV40 has no such effect (Morimoto and Milarski, 1990; Jindal and Young, 1992). Vaccinia virus infection leads ultimately to an association of a high percentage of the total pool of Hsp70 with viral proteins, and appears to enhance stability of Hsp70, Hsp60 and Hsp90 mRNA relative to other cellular mRNAs (Jindal and Young, 1992). Interestingly, no association between viral proteins and Hsp60 was found, Hsp90 has been found on cell surface membranes after infection with herpes virus, and the protein is induced at much greater levels in viral infection than

heat shock (La Thangue and Latchman, 1988). Cell lines expressing low levels of Hsp90 were less thermotolerant than control lines, but no difference in their sensitivity to herpes simplex virus infection was observed, arguing against a simple protective role for this molecule (Bansal *et al.*, 1991). In those cases where host hsp gene expression is induced a specific viral gene may be responsible for generating a heat shock response, implying a fundamental need for these proteins in viral infections. For example, the 12S and 13S E1A gene products are responsible for induction of an Hsp70 gene in adenovirus infection (Neidhardt *et al.*, 1984; Simon *et al.*, 1987).

1.2.4. Hsps and cancer

One field of heat shock protein biology currently receiving a great deal of attention concerns the role of these molecules in tumour development. It is beyond the scope of this thesis to discuss fully this fascinating topic, and it will thus be only briefly touched on.

In a study of six tumour cell lines, there was general overexpression of cytoplasmic Hsp90, and some lines also overexpressed Hsp72 in the absence of environmental stress (Ferrarini *et al.*, 1992). In contrast to normal cells, Hsp72 expression was not cell cycle-regulated, and Hsp72 was found to be overexpressed in fresh tumour cells. Furthermore, some surface expression of both hsps was reported in these lines.

Breast cancers are known to be responsive to estrogen, and since Hsp90 is associated with estrogen receptors, the role of this molecule in breast cancer has come under scrutiny. Hsp90α was shown to be overexpressed in breast cancer cells, although no association between the levels of estrogen receptor and Hsp90 expression was observed (Jameel *et al.*, 1992). Overexpression of Hsp90 correlated with occurrence of lymph-node metastases, and hence with a worse long-term prognosis (Jameel *et al.*, 1993). In a study of sera from 125 malignant

41

breast cancer patients, 46 (37%) had anti-Hsp90 autoantibody levels greater than the mean plus three standard deviations of controls (Conroy et al., 1995). In general, high autoantibody levels correlated with the development of metastases.

Elevated Hsp90α levels have been documented in acute leukaemic cells from cancer patients, while the same study revealed that leukaemic cell lines overexpressed both Hsp90α and Hsp90β (Yufu et al., 1992). Overexpression of Hsp27 was also detected in approximately 50% of patients with lymphoblastic leukaemia. In a survey of pancreatic carcinomas hsps showed differential expression, with Hsp90α selectively overexpressed in tumour cells, although an increase in Hsp72 mRNA was also noted (Gress et al., 1994). In this study, Hsp90β and ubiquitin were found constitutively at high levels in all tissues examined, and appeared to be unrelated to the tumour state. The majority of lung cancers infiltrated by γδ T lymphocytes, but not those infiltrated by αβ T cells, had increased expression of Hsp72 (Ferrarini et al., 1994).

The importance of these findings can be seen in experiments showing that hsps can elicit specific immunity to tumours from which they originate. A comparison of tumour-specific immunogenicities of Hsp90, the closely-related gp96 protein (Ullrich et al., 1986) and Hsp70 revealed that Hsp70 and gp96 were highly and equally immunogenic, while Hsp90 had only 10% of the immunogenicity of the other two molecules (Udono and Srivastava, 1994). This may be a function of molecular chaperoning properties of hsps, and it has been suggested that hsps are not immunogenic per se, but serve as carriers of highly antigenic low molecular weight molecules (Srivastava, 1993). Such molecules have been found associated with gp96 (Li and Srivastava, 1993). The different immunogenicities observed could thus result from compartmentalisation of the respective hsps, with cytosolic Hsp90 having less access to the more highly antigenic peptides than endoplasmic reticulum-associated gp96 or the widely distributed Hsp70. It is interesting to note, therefore, that a heat-inducible 70kD protein is a strong

candidate for a tumour rejection antigen in transformed murine fibroblasts (Tamura *et al.*, 1993). Grp78 may also have a protective role in tumour growth, as induction of this protein correlates with the development of resistance to lysis by cytotoxic T cells (Sugawara *et al.*, 1993). This group also reports that the phosphorylation state of grp78 is important in conferring resistance to T cell cytotoxicity, and that the T lymphocytes themselves express high levels of grp78, perhaps utilising a similar mechanism to avoid autolysis.

A novel approach to cancer therapy has recently been tested by vaccinating mice with Hsp90, Hsp70 and gp96 derived from methylcholanthrene A sarcoma cells (Blachere *et al.*, 1993). In these experiments vaccination with hsps afforded protection against the specific tumour from which the hsp was derived but no protection from antigenically distinct tumours, in line with the notion of these proteins as antigen carriers. Gp96-induced immunisation appears to be linked to CD8$^+$ priming, as populations depleted of these cells abrogated the gp96-elicited immunity (Udono *et al.*, 1994).

1.2.5. Hsps and autoimmunity

There are several lines of argument implicating a role for hsps in autoimmune disease (Kaufmann, 1994):

(1)   Autoimmune disease has developed in experimental models following immunisation with hsps.

(2)   Hsp-reactive T cells have been isolated from lesions of arthritis patients.

(3)   T cells with specificity for bacterial hsps have been obtained from patients with various autoimmune diseases.

(4)   There is an ever-increasing list of diseases in which anti-hsp antibodies have been discovered.

(5)   Elevated Hsp levels have been described in inflammatory lesions of a range of autoimmune diseases.

Perhaps the best known example of hsp involvement in autoimmune disease is in adjuvant arthritis in Lewis rats. This disease is used as a model for rheumatoid arthritis in humans and is characterised by an inflammatory reaction in synovial tissue, leading to destruction of the joints. Adjuvant arthritis is induced by immunisation with heat-killed *Mycobacterium tuberculosis* in oil, and the epitope found to drive the immune response has been identified as a nine amino acid sequence (180-188) of mycobacterial Hsp65 (Van Eden *et al.*, 1988; Van der Zee *et al.*, 1989; Fu *et al.*, 1994). Two amino acids in this peptide, phenylalanine in position 181 and leucine in position 183, are required for stimulatory activity of $\gamma\delta$ T cells (Fu *et al.*, 1994).

The disease is transferable, with T cells specific for this antibody capable of generating new disease in fresh rats. However, certain Hsp65 antigen-driven T cell clones may have an opposite effect upon transfer, and play a protective role in adjuvant arthritis, ameliorating or preventing the condition (see Feige and Cohen, 1991). Pretreatment with Hsp65 boosts resistance to arthritis induction, and different strains of rats respond to varying degrees to the mycobacterial Hsp65 180-188 epitope in a manner that corresponds with arthritis susceptibility. Furthermore, a role for Hsp65-specific T cells in controlling T cell responses to epitope 180-188 has been described (Hogervorst *et al.*, 1991). An investigation into cellular and humoral responses in Lewis rats revealed Hsp65 but not Hsp70 to be an immunodominant antigen of *M. tuberculosis*, with antibodies to Hsp65 observed two weeks after immunisation with heat-killed mycobacteria (Hogervorst *et al.*, 1992). High levels of Hsp65 antibodies corresponded to a low arthritis score, i.e. low disease activity, a finding which has been corroborated by studies on non-arthritic Wistar rats, in which higher titres of anti-Hsp65 antibodies were noted in the sera of these animals than in rats that developed arthritis (Ramos-Ruiz *et al.*, 1991).

Administration of Hsp65 during disease development resulted in deterioration of the condition and coincided with raised T cell responsiveness to Hsp65 and the

180-188 epitope. However, injection of epitope 180-188 or purified Hsp65 is not by itself arthritogenic, and pretreatment with an immunogenic form of the protein 3-4 weeks before arthritogenic challenge completely protects against adjuvant arthritis (Billingham *et al.*, 1990). A recent paper investigating T cell epitopes of Hsp65 identified nine peptide sequences that induced T cell responses in Lewis rats, including peptide 180-188 (Anderton *et al.*, 1994). A significant difference between the patterns of dominance of epitopes recognised by these rats after immunisation with *M. tuberculosis* or with purified Hsp65 was noted, and two co-dominant epitopes were recognised on immunisation with the purified protein that were recognised only weakly by mycobacterial-immunised rats. Interestingly, of the nine sequences studied, only one minor epitope was cross-reactive with rat Hsp60.

Other forms of experimental arthritis have been shown to be inhibited by pretreatment with Hsp65, including streptococcal cell wall arthritis in rats and pristane-induced arthritis in mice (Van den Broek *et al.*, 1989; Thompson *et al.*, 1990). However, evidence for cross-reactivity or molecular mimicry between mycobacterial Hsp65 and mammalian Hsp60 is weak, there is no homologous epitope to peptide 180-188 in human Hsp60, and development of an autoimmune response to Hsp65 does not necessarily lead to an autoimmune disease, calling into question whether conclusions drawn from the adjuvant arthritis model are relevant to rheumatoid arthritis (Yang and Feige, 1991; Anderton *et al.*, 1994). Furthermore, adjuvant arthritis is mostly a self-limited condition, whereas rheumatoid arthritis may last for decades. Conflicting evidence has been presented regarding involvement of Hsp60 in rheumatoid arthritis, and the disease is undoubtedly more complex than a simple hypothesis of autoreactive attack by γδ T cells against a conserved Hsp60/cartilage cross-reactive epitope. Hsp65 does not appear to be an immunodominant antigen recognised on bulk level by synovial fluid

mononuclear cells and the response to mycobacterial Hsp65 decreases with increasing purification of the protein (Life et al., 1991; Res et al., 1991).

A second experimental autoimmune disease in which some evidence exists for Hsp60 involvement is insulin-dependent diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice. This disease develops spontaneously in these mice from the age of 4-6 weeks and is due to progressive destruction of insulin-producing beta cells. The critical (overt) stage is reached at 4-6 months, whereby mice are unable to produce sufficient insulin for survival. Hsp60 may be the target self-antigen for T cells to destroy the mouse's own beta cells. An Hsp60-related antigen is found in blood of NOD mice approximately two months prior to overt disease, and has been termed cross-reactive antigen (Elias et al., 1990). This is followed by generation of anti-Hsp60 antibodies and Hsp60-reactive T cells. These T cells are much more specific for mammalian Hsp60 than mycobacterial Hsp65 and by transfer of these T cells into prediabetic recipient mice and non-diabetic mice it has been shown that these cells are sufficient to cause IDDM. Furthermore, immunisation experiments have shown that immunity to Hsp60 appears to be necessary as well as sufficient for the development of IDDM (Cohen, 1991). Other researchers have failed to replicate these results, however, and have questioned these findings (Kämpe et al., 1990; Atkinson et al., 1990; Latchman, 1991). An alternative candidate as the autoantigen responsible for generating autoimmune disease has emerged, namely the 64-kD glutamic acid decarboxylase protein (Baekkeskov et al., 1990; Kämpe et al., 1991).

Evidence has also been presented implicating Hsp60 in the pathogenesis of multiple sclerosis. In acute multiple sclerosis lesions there is an accumulation of γδ T cells, seemingly as a result of clonal expansion in multiple sclerosis plaques due to increased expression of Hsp60 and Hsp90 (Wucherpfennig et al., 1992). Hsp60 is expressed on large, reactive, proliferating oligodendrocytes at the edge of chronic multiple sclerosis lesions, and colocalises with γδ T lymphocytes (Selmaj et al., 1991). Hsp72 expression in the same cells is only expressed at low

levels, implying that this phenomenon cannot be regarded as a simple stress response. Hsp65-expressing oligodendrocytes are immature, with strong reactivity for myelin basic protein, and it has been postulated that $\gamma\delta$ T cells reactive to Hsp60 may prevent remyelination in multiple sclerosis lesions (Selmaj et al., 1992). Antibodies to Hsp60 were found at similar levels in the cerebrospinal fluid of multiple sclerosis patients and patients with other neurological disorders, arguing against a specific humoral response to Hsp65 in this disorder (Gao et al., 1994).

In humans, T cells that recognise Hsp65 have been identified in the peripheral blood and synovial fluid of reactive arthritis, juvenile chronic arthritis and rheumatoid arthritis patients (Life et al., 1991; De Graeff-Meeder et al., 1991; Danieli et al., 1992). Patients with juvenile chronic arthritis had antibody titres to Hsp65 that were generally higher with clinically active disease, but adult rheumatoid patients responded poorly to Hsp65, and not at all to peptide 180-188 (De Graeff-Meeder et al., 1991; Danieli et al., 1992). However, synovial fluid-derived T cells from patients with juvenile chronic arthritis showed a greater response to Hsp65 than T cells derived from peripheral blood, but little or no response to pure human Hsp60 (Life et al., 1993).

High Hsp72 levels have been observed in the autoimmune thyroid diseases Graves' opthalmopathy and Hashimoto's thyroiditis compared with multinodular goitre and normal controls. This elevation is unrelated to changes in thyroid hormone levels (Heufelder et al., 1991; Heufelder et al., 1992a). Interestingly, Hsp72 is found on the cell surface of routinely cultured retroocular fibroblasts from patients with Graves' opthalmopathy and persists in culture despite the absence of immunological stress, suggesting more than just a short-lived stress-induced phenomenon (Heufelder et al., 1992b).

Lyme arthritis is caused by a tick-borne spirochete, Borrelia burgdorferi In a limited study, 4/5 patients with Lyme arthritis were shown to be capable of producing T cells and antibodies that reacted with recombinant B. burgdorferi

Hsp60, while no reactivity was observed in healthy controls. These patients appeared to recognise a unique epitope, as a related human Hsp60 epitope failed to generate any reactivity (Shanafelt *et al.*, 1991).

In a comprehensive survey of hsp protein expression in peripheral blood mononuclear cells (PBMCs) of patients with rheumatic disease, significantly elevated Hsp90 levels were noted in patients with dermato/polymyositis, systemic sclerosis and multiple sclerosis. Hsp72 overexpression was observed in patients with ankylosing spondylitis, while Hsp60 overexpression was observed only in patients with Behçet's syndrome. No overexpression of Hsp73 was noted in any disease group, although there appeared to be decreased levels of this protein in those with Behçet's syndrome, rheumatoid arthritis, Sjögren's syndrome and multiple sclerosis (Dhillon *et al.*, 1993a). Levels of Hsp90 in patients with Behçet's syndrome were slightly higher than that of healthy controls, but this failed to reach statistical significance, although several individuals had protein levels well above the control range (Dhillon *et al.*, 1992; Dhillon *et al.*, 1993a). IgG and/or IgM autoantibodies to Hsp60 have been found in sera of patients with mixed connective tissue disease, polymyositis/dermatomyositis and psoriatic arthritis, while autoantibodies to Hsp73 have been noted in patients with Lyme arthritis (Jarjour *et al.*, 1991). Little or no autoantibodies were detected in sera from patients with Sjögren's syndrome, rheumatoid arthritis, ankylosing spondylitis or control sera, and were found infrequently in systemic sclerosis patients.

In contrast, studies using more sensitive ELISA systems have detected autoantibodies to human Hsp60 in nearly all sera from patients with rheumatoid arthritis and mixed connective tissue disease, as well as elevated antibodies to Hsp73 in rheumatoid arthritis and mixed connective tissue disease (Mairesse *et al.*, 1993). Anti-Hsp60 antibodies have also been discovered in patients with juvenile chronic arthritis or cystic fibrosis but not in patients with IDDM (De Graeff-Meeder *et al.*, 1993). The different methods used in these

studies may account for the seemingly contradictory results, with the more sensitive ELISA able to detect low level titres of antibodies that a less sensitive immunoblotting assay would miss.

That heat shock proteins are abnormally expressed in at least some autoimmune disorders is clear, but whether they are directly or indirectly involved in pathogenesis has yet to be decisively proved. Despite the large number of autoimmune diseases with hsp-reactive T cells, antibodies or autoantibodies there is little evidence, with the possible exception of Hsp65 in multiple sclerosis that these T cells or antibodies are involved in autoimmune tissue damage. However, using Hsp60-specific anti-sense oligonucleotides to reduce the synthesis of Hsp60 in Schwann cells autoimmune lysis of these cells and macrophages by cytotoxic T lymphocytes was inhibited, arguing for at least some role in the autoimmune process (Steinhoff *et al.*, 1994). Despite this, the case for active participation of hsps and antibodies to them in autoimmune disease remains unproved.

1.3: Systemic Lupus Erythematosus (SLE)

1.3.1. SLE

Systemic Lupus Erythematosus (SLE) is a disease of unknown aetiology affecting primarily women of child-bearing age. A milder disease can be induced by a number of drugs and may account for up to 10% of lupus cases. The female:male ratio is approximately 9:1, although there is a subset of SLE patients in which the disease appears to be mainly male, transmitted from father to son. Estimates of prevalence range from 1 in 250 women in Jamaica to 1 in 4312 women in New Zealand, and there are undoubtedly ethnic differences in incidence, reflecting a genetic component. Virtually any organ may be involved in the disease, though polyarthralgia and skin rashes occur in most patients (reviewed in Worrall *et al.*, 1990; Isenberg and Horsfall, 1993). The revised criteria used for the classification of SLE by the American Rheumatism Association is given in Table 1.3.

The prognosis for SLE patients has improved with advent of earlier diagnosis, increased recognition of mild cases and more cautious use of corticosteroids. Survival rates of 90% over 10-years (Talal, 1992), and 88% over 5 years (Worrall *et al.*, 1990) appear to be typical figures reported by SLE clinics, although juvenile SLE may be particularly severe and the survival rate in these cases is somewhat lower (Morrow and Isenberg, 1987). The most common causes of death are renal disease and infection, and SLE patients may have a slightly increased risk of developing cancer, particularly lymphoma (Pettersson *et al.*, 1992; Menon *et al.*, 1993).

SLE is characterised by a range of serological abnormalities, including autoantibodies to DNA, RNA and other anti-nuclear antigens (ANAs). The presence of anti-double-stranded DNA (anti-dsDNA) antibodies is probably the single most important laboratory criterion for diagnosis of the disease, around

75% of patients being seropositive. Other autoantibodies commonly found in SLE include antibodies to RNP, Sm, Su, ribosome phosphoproteins, SS-A(Ro), and SS-B(La), while rheumatoid factor (antiglobulins) is also found frequently in SLE patients (Worrall *et al.*, 1990). Furthermore, spontaneous polyclonal B cell activation is increased (Cohen, 1993). However, it should be noted that raised autoantibody levels do not necessarily correlate with disease activity (Isenberg *et al.*, 1984).

T cells are regarded as being central to the pathogenesis of SLE. The presence of activated T cells, including T cells expressing MHC class II molecules, has been noted, and these T cells may be involved in mediation of autoimmune tissue damage (Cohen *et al.*, 1982; Caligaris-Cappio *et al.*, 1985). A decrease in the ratio of $CD4^+/CD8^+$ cells has also been reported, either through steroid therapy or by an unrelated mechanism (Bakke *et al.*, 1983). T cells from SLE patients exhibit increased expression of a number of activation markers, as well as Il-2 receptors (Alcocer-Varela *et al.*, 1991). The involvement of cytokines in the disease remains uncertain, but a role for these molecules is likely. For example, accessory cells in SLE patients do not appear to produce sufficient Il-1 for T cell activation, although this may be due to a defect in the Il-1 receptor rather than through a flaw in the mechanisms generating cytokines (Isenberg and Horsfall, 1993).

A genetic component to SLE has been identified, and there appears to be an association between the disease and class II MHC genes, particularly HLA-DR2 and HLA-DR3. In one clinic the A1, B8 haplotype was associated with an eight-fold relative risk amongst the SLE patients who were Caucasian in origin, and an even greater risk was shown for patients with B8, DR3. However, as those researchers pointed out, HLA associations should be treated with caution, since studies with different ethnic compositions have shown different trends (Worrall *et al.*, 1990).

Other studies have also suggested that MHC class II associations are linked to autoantibody subsets in patients with SLE. There is, for example, an apparent link between high titres of anti-dsDNA antibodies and HLA-DR2 and HLA-DR3, although more recent evidence suggests that these autoantibodies may be more strongly linked to HLA-DQw2.1 (Reveille, 1992). Given the putative functions of MHC class I and class II heterodimers in antigen processing and presentation to receptors on CD4$^+$ T cells links between autoantibodies and HLA-type are not surprising. Perhaps the strongest genetic association lies in the MHC class III region, where a strong correlation exists between increased frequencies of C4 null alleles and SLE, and the percentage of patients with partial C4A deficiency, i.e. having one dysfunctional allele, is significantly increased in SLE. There may be as many as 80% of SLE patients who have a partial C4 deficiency, although there is a significant percentage of healthy controls with similar deficiencies. In addition to C4, other deficiencies of the early classical pathway of complement components are increased in frequency in SLE, particularly C1 and C2 (reviewed in Reveille, 1992; Isenberg and Horsfall, 1993). The involvement of the complement receptor has also been considered, and low expression of this receptor on erythrocytes and peripheral blood leukocytes has been described in patients with SLE and members of their families (Walport et al., 1988).

SLE is thus a heterogeneous disease which presents in a variety of forms, and may even be considered as a set of related conditions brought together under one "disease label". Mortality rates have fallen somewhat, but there remains considerable morbidity associated with the disease, although some of the depression and anxiety felt by these patients may be the psychological effect of having a chronic and unpredictable disease.

Table 1.3. Defining symptoms for classification of SLE

1	Malar rash
2	Discoid rash
3	Photosensitivity
4	Oral ulcers
5	Arthritis
6	Serositis
	a) pleuritis or
	b) pericarditis
7	Renal disorder
	a) proteinuria > 0.5g/24hr or 3+, persistently or
	b) cellular casts
8	Neurological disorder
	a) seizures or
	b) psychosis (having excluded other causes, e.g. drugs)
9	Haematological disorder
	a) haemolytic anaemia or
	b) leucopenia or < 4.0 x 10^9/l on two or more occasions
	c) lymphopenia or < 1.5 x 10^9/l on two or more occasions
	d) thrombocytopenia < 100 x 10^9/l
10	Immunologic disorders
	a) positive LE cell or
	b) raised anti-native DNA antibody binding or
	c) anti-Sm antibody or
	d) false positive serologic test for syphilis, present for at least six months
11	Anti-nuclear antibody in raised titre

Table 1.3. To diagnose SLE it is necessary for four or more of the 11 criteria listed above to be present, either at once or serially, during any observational period (Tan *et al.*, 1982).

1.3.2. Murine models of SLE

Investigation of SLE pathogenesis and aetiology has been aided by development of a range of animal models. The New Zealand Black (NZB) mouse and the NZB/W mouse, the F1 offspring from a cross between the NZB mouse and the New Zealand White (NZW) mouse, were the first models that spontaneously developed SLE-like illnesses, but the range of murine lupus models has expanded to include MRL/MpJ-*lpr/lpr* (and its congenic strain MRL/MpJ-+/+), BXSB, SWR/NZB, Palmerston North, Swan, and Motheaten mice, all of which develop at least some characteristics of SLE disease. In addition, the *lpr* gene has been transferred to a variety of other strains, and a second disease, *gld* (for generalised lymphocytic disorder) has been characterised which gives rise to symptoms identical to *lpr* in mice with identical backgrounds, but is non-allelic (Roths *et al.*, 1984). Furthermore, a second mutation in the *lpr* gene, *lpr^cg*, has been discovered (Matsuzawa *et al.*, 1990). Finally, increasing development and availability of transgenic models has enabled researchers to investigate specific features that may be relevant in the pathology of SLE.

The NZB strain, which inherits haemolytic anaemia, was bred over 35 years ago at the University of Otago in New Zealand (Bielschowsky *et al.*, 1959). Cross-breeding of this strain with a phenotypically normal NZW strain at the same institution gave rise to the first true murine model for SLE, the NZB/W mouse (Helyer and Howie, 1963). This mouse has been the focus of intense research and much useful information has been obtained in that time. With only one strain available, however, it was impossible to determine the relevance of some of the unusual features seen in these mice, and development of two new strains of mice which also spontaneously develop SLE-like diseases, MRL/MpJ-*lpr/lpr* (MRL/*lpr*) and BXSB strains, each with a unique genetic background, has greatly enhanced the utility of these experimental SLE models (Murphy and Roths, 1978).

NZB/W, BXSB and MRL/*lpr* mice share a variety of abnormalities which can be regarded as characteristic of murine SLE, including glomerulonephritis, thymic atrophy, lymph node hyperplasia, and development of anti-DNA antibodies. However, the MRL/*lpr* mouse has several advantages as an experimental tool over other strains. Firstly, MRL/*lpr* has a congenic strain, MRL/MpJ-+/+ (MRL/++), which develops a mild form of disease later in life. The MRL/++ strain shares >99% genetic identity with MRL/*lpr*, with the severe disease seen in the latter strain arising from a defect in a single autosomal recessive gene, termed *lpr* (for lymphoproliferation). Second, both males and females develop disease in MRL/*lpr* mice, although the disease appears to be more acute in females, with a concomitant decrease in life expectancy. In contrast, NZB/W males and BXSB females appear to develop only mild disease and have a greatly enhanced lifespan compared with their opposite genders. Third, several serological features of MRL/*lpr* disease which closely mimic symptoms of human SLE appear to be unique among murine models, including generation of anti-Sm (Eisenberg *et al.*, 1978), anti-ribosomal P protein (Bonfa *et al.*, 1988) and anti-Su (Treadwell *et al.*, 1993) antibodies. However, MRL/*lpr* mice develop massive lymphadenopathy, a symptom not generally paralleled in human patients.

The *lpr* gene has recently been shown to encode for the *Fas* antigen which mediates apoptosis (Watanabe-Fukunaga *et al.*, 1992a), prompting a flurry of experimental activity with MRL/*lpr* and other *lpr* gene-bearing mice. The significance of this finding is discussed in more detail below.

1.3.3. The MRL/*lpr* mouse

The MRL (H-2^k) strain evolved from a series of crosses involving four inbred strains, LG/J (75%) C3H/Di (12.1%), AKR/J (12.6%) and C57BL/6J (0.3%), originally bred for studying leukaemia. After several generations of inbreeding a proportion of mice in the new MRL strain developed massive generalised lymph

55

node enlargement, while other mice appeared to remain phenotypically normal. These two subsets were divided and subsequently inbred, giving rise to MRL/l mice, which developed enlarged lymph nodes, and MRL/n which did not. Isolation of the *lpr* gene enabled backcrossing of the mutant gene from MRL/l onto MRL/n mice, which were renamed MRL/MpJ-*lpr*/*lpr* and MRL/MpJ-+/+ mice respectively. The residual genetic difference between MRL/*lpr* and MRL/++ mice is less than 0.1%, and the two strains are generally believed to be identical apart from the mutated *lpr* gene. The phenotype of MRL cell surface alloantigens include Ly-1.2, Ly 2.1, Ly 3.1, Thy 1.2, TL⁻ and Qa-$1^{b..}$ The allotype of the IgG_{2a} subclass for MRL strains is a (Murphy and Roths, 1978).

There is 50% mortality by five months in most colonies, MRL/*lpr* females having a mean survival time of 143 days, slightly less than that of males, which have an average lifespan of 154 days. This compares with mean survival times of 476 and 546 days for MRL/++ females and males (Theofilopoulos and Dixon, 1985). The major cause of death in all lupus mice is renal disease.

1.3.4. Morphological features of the MRL/lpr disease

Glomerulonephritis. The terminal glomerulonephritis MRL/*lpr* mice develop in the course of disease is largely a subacute proliferative form, with accumulation of monocytes and proliferation of both endothelial and mesangial cells. There is occasional crescent formation and basement membrane thickening. Some proteinuria is evident; typically MRL/*lpr* males and females between 3 and 6 months of age have urinary protein values from 2.6-3.8 mg/day (Andrews *et al.*, 1978). Granular IgG and complement C3 deposits also increase from 2 to 5 months. The IgG eluted from MRL/*lpr* kidneys is predominantly IgG_{2a} and IgG_{2b} subclasses. The concentration of antinuclear antibodies (ANA) in IgG eluted from kidneys is 2-4 times higher than that found in serum IgG, and anti-dsDNA antibodies are found at even greater relative concentrations.

Thymic Atrophy. In all lupus mice strains, slightly more than 90% of animals have severe cortical thymic atrophy (Andrews *et al.*, 1978). However, a small percentage of mice develop medullary hyperplasia which maintains or increases overall thymus size despite the cortical loss. This hyperplasia does not correlate with other features of disease. In MRL/*lpr* mice thymic atrophy appears by 2 months and leads to a complete loss of cortical area by 3.5 months. MRL/*lpr* animals thymectomized at day one and transplanted at one month of age with a MRL/++ thymus retained the disease phenotype of unmanipulated MRL/*lpr* mice. Likewise, transplantation of MRL/*lpr* thymuses to MRL/++ mice failed to accelerate the disease in those animals. MRL/*lpr* mice thymectomized but not transplanted did not develop lymphoid hyperplasia and autoimmune disease, and also had greatly reduced levels of serum IgG and anti-DNA antibodies (Theofilopoulos and Dixon, 1981). From these results the authors concluded that T cell differentiation in the thymus was a necessary component of the MRL/*lpr* phenotype.

Vascular disease and myocardial infarction. 15-30% of MRL/*lpr* mice have had myocardial infarcts sufficiently extensive enough to be judged a contributing cause of death. Three-quarters of older MRL/*lpr* mice have necrotizing exudative polyarteritis, involving mostly medium-sized arteries of the kidney, genitals and heart (Andrews *et al.*, 1978). This feature is associated with high autoantibody levels and circulating immune complexes.

Lymph node enlargement. Massive lymphoid hyperplasia is among the most striking features of the MRL/*lpr* disease, and lymph nodes may be 100-fold greater in size than normal mice. This lymphoid enlargement commences by 8 weeks of age, progressing to the grossly abnormal size described by 16-18 weeks (Andrews *et al.*, 1978), the increase being more marked in females than males. A single node may predominate somewhat, and may reach 2200mg (compared with 50mg for similar normal nodes). The origin of the increase in size is primarily lymphocytic, and comprises plasma cells, histiocytes and

immunoblasts, although the primary cell type is $CD4^-CD8^-Thy1^+$, the so-called double negative cell. Although the number of $CD4^+$ or $CD8^+$ cells increases in absolute number relative to normal mice, the huge number of double negative cells means that the proportion of single positive T cells may drop to less than 10% of cells in the lymph node (Wofsy et al., 1984). Normal nodal architecture of the lymph is destroyed by mass production of these cells. Plasma cell density increases from about 3 months, and large groups of plasma cells develop in the medulla with lymphoproliferation. In some older MRL/lpr mice there is evidence of cystic necrosis and haemorrhage and this may account for a reduction in lymph node weight compared with maximum seen in some mice in the latter stages of disease. Fewer than 5% of lymph cells in enlarged nodes are proliferating (Raveche et al., 1982), a similar percentage to that seen in control mice. However, the absolute number of proliferating cells is greatly increased as a result of increased lymph size. MRL/++ mice do not develop this lymphoproliferative feature. Attempts to transplant enlarged lymph nodes to find evidence of malignancy in MRL/lpr mice have failed (Theofilopoulos and Dixon, 1985), although there are abnormal expression patterns of several oncogenes in these mice (Cohen and Eisenberg, 1991).

Splenomegaly. There is an approximate 7-fold enlargement of spleens of older MRL/lpr compared with age-matched controls (Theofilopoulos and Dixon, 1985).

Arthritis. MRL/lpr is unique among lupus strains, as 20-25% of older mice develop swelling of the joints and surrounding tissues of hind feet and lower legs (Andrews et al., 1978). Histological analysis shows that approximately 75% of 5-6 month old mice have significant joint pathology (Hang et al., 1982). This mouse is the only animal with spontaneous arthritic symptoms, and hence has also been widely used as a model for rheumatoid arthritis. However, the percentage of animals in different colonies developing arthritic symptoms is variable and unpredictable.

Other histopathological characteristics. Conjunctivitis and mononuclear infiltrates in lachrymal glands have been reported in MRL/*lpr* mice (Hoffman *et al.*, 1984). Over 60% of older MRL/*lpr* mice also exhibit salivary gland mononuclear infiltration, and this mouse has thus also been utilised as a model for Sjögren's syndrome.

1.3.5. Serological features of the MRL/*lpr* disease

Immunoglobulins. All lupus mice have significantly higher total polyclonal IgG concentrations at preclinical or clinical stages than normal mice. In the MRL/*lpr* mouse a four-fold increase over normal is observed at 2 months, rising to 10-13 times normal levels by 4-5 months of age. Increases are predominantly in the IgG_1, IgG_{2a} and IgG_{2b} subclasses. Serum IgM levels also rise approximately three-fold compared with controls. There is a relatively high incidence of monoclonal γ-globulins in MRL/*lpr* mice by 5-6 months, with 53% of females and 29% of males having monoclonal immunoglobulin. There is no apparent correlation between appearance of restricted immunoglobulin bands and levels of polyclonal IgG in serum, or with levels of autoantibodies. There is a slight correlation between presence of monoclonal immunoglobulin and other clinical features, including glomerulonephritis and necrotizing vasculitis. However, these monoclonal immunoglobulins have not been isolated from kidney eluates. No restricted Ig bands were found in sera from MRL/++ mice, nor were such bands detected in sera from MRL/*lpr* x MRL/++ F1 crosses (Theofilopoulos and Dixon, 1985).

Anti-DNA antibodies. Production of anti-DNA antibodies is characteristic of human SLE and murine lupus strains, and much research has focused on this feature. Anti-ssDNA antibodies are found at low levels in immunologically normal mice, but greater levels of these antibodies are observed at two months in MRL/*lpr* mice, and these levels increase with age. Anti-dsDNA antibodies

develop later in the course of disease, but significantly elevated antibody levels are seen by 4-5 months. Generation of these antibodies appears to be linked with spontaneous polyclonal B cell hyperactivity (Pisetsky *et al.*, 1980; Dixon, 1981). DNA deposits and anti-DNA antibodies are almost certainly involved in the pathogenesis of MRL/*lpr* disease. High concentrations of anti-DNA antibodies are found in their kidney eluates, and studies on F_1 crosses have shown a direct relationship between anti-DNA antibody levels, glomerulonephritis and mortality. Interestingly, antibodies to Z-DNA and RNA have also been detected in MRL/*lpr* mice; their relevance to the disease process is unknown.

Anti-Sm antibodies: Generation of autoantibodies to the nuclear glycoprotein Sm is a characteristic of human SLE. Unique among lupus-prone strains, a subset of MRL/*lpr* and MRL/++ mice also generate anti-Sm autoantibodies. In 4-5 month-old animals, 37% of male MRL/*lpr* mice and 10% of females generated positive antibody responses to this antigen (Eisenberg *et al.*, 1978), although higher percentages of antibody-positive mice have been reported (Pisetsky *et al.*, 1980). Antibodies to Sm were undetectable in MRL/*lpr* mice younger than four months, although a small percentage of MRL/++ mice generated anti-Sm antibodies at 1-3 months (Eisenberg *et al.*, 1978). In MRL/++ mice 83% of females and 57% of males had detectable anti-Sm antibodies at 9-12 months of age. No clinical features have been linked with the appearance of these antibodies, and this feature does not correlate with B cell hyperactivity.

Antiretroviral gp70 autoantibodies. Although high levels of serum gp70 are found in both lupus strains and immunologically normal mice, only the former spontaneously produce antibodies to this molecule. Gp70/anti-gp70 complexes are found in sera of all lupus prone mice and their appearance parallels onset of renal disease. Levels of antibody-bound gp70 increase with disease progression in MRL/*lpr* mice, although total amount of the protein in serum remains relatively unchanged from 2 months of age. However, MRL/*lpr* sublines with low

gp70 and related immune complex levels have an essentially identical disease profile, calling into question the importance of these antibodies in the MRL/*lpr* disease (Theofilopoulos and Dixon, 1985).

Rheumatoid factors. As mentioned above, a proportion of MRL/*lpr* mice develop arthritic symptoms similar to human rheumatoid arthritis. In support of these findings, nearly 50% of these mice develop IgG and IgM rheumatoid factors at 3-4 months of age.

Other serological abnormalities. In MRL/*lpr* mice increased levels of cryoglobulins are found to correlate with age and disease severity, values increasing from 170µg/ml at 2 months to >2000µg/ml at 5 months (Andrews *et al.*, 1978). In addition, concentrations of haemolytic complement fall with disease onset and progression in all lupus strains of mice.

1.3.6. Cellular abnormalities in MRL/*lpr* mice

The capacity of MRL/*lpr* mice to respond to exogenous immune stimulation is impaired. The antibody response to foreign antigens and polyclonal B-cell activators appears to be defective.

B cell abnormalities. All lupus strains spontaneously produce more anti-hapten antibody secreting cells in spleen and greater concentrations of anti-hapten antibodies in sera than age-matched immunologically normal strains (Izui *et al.*, 1978), correlating with spontaneous development of anti-ssDNA antibodies. A high frequency of immunoglobulin-secreting cells is first observed around the time of disease onset (Theofilopoulos and Dixon, 1981; Dixon, 1981). Late in the course of disease MRL/*lpr* mice have a 30-fold higher number of immunoglobulin-containing cells than younger animals. Spleens from young mice produce predominantly IgM, but switch to IgG with age and disease onset. The spleens of MRL/*lpr* mice have higher percentages and absolute numbers of spontaneously proliferating cells at disease onset than normal mice, but this

increase is not matched in thymuses or bone marrow of these animals (Raveche et al., 1982). The enhanced production of IgG appears selective for IgG subclass. In MRL/lpr females from 2-5 months of age there is an eight-fold increase in number of cells producing IgG_{2a}, a six-fold increase in cells producing IgG_{2b} and smaller increases in IgG_1- and IgG_3-secreting cells (Slack et al., 1984).

Spontaneous polyclonal B cell activation does not account for all autoantibody subsets and titres observed, and some antibody profiles, such as the anti-Sm response, appear to have an antigen-driven mechanism (Klinman et al., 1990).

T cell abnormalities. Immunoglobulin hypersecretion, autoantibody production and hyperresponsiveness to exogenous antigens may be caused by a decrease in suppressor T cell activity or an increase in helper T cell activity. There is conflicting evidence regarding the role of suppressor T cells in pathogenesis of murine lupus, but T cell-enriched populations from old MRL/lpr mice were shown to provide greater help to B cells from syngeneic young animals than young MRL/lpr mice or haplotype-matched controls (Theofilopoulos and Dixon, 1981). The T cell excess used in these experiments was far less than that seen in vivo and enhanced helper activity is probably even greater in intact animals. Interestingly, a converse situation appears to apply in human SLE, where half of the patients in one study had markedly depressed percentages and absolute numbers of cells expressing the T inducer/helper marker OKT4 (CD4), and increased numbers and percentages of cells expressing the T cytotoxic/suppressor marker OKT8 (CD8). Low OKT4/OKT8 ratios were associated with a serological activity index comprising low serum complement, cryoglobulins, high anti-DNA titres and anti-DNP titres (Bakke et al., 1983).

Cultured cells of MRL/lpr mice can begin producing B Cell Differentiation Factor (BCDF), a molecule that can induce proliferation of activated B cells, from as young as one month, but levels increase with age and onset of lymphoproliferation. This factor is produced by T cells of the Lyt-1^+ phenotype, which is the dominant cell in the enlarged MRL/lpr lymph nodes and spleen.

Interestingly, a study investigating pathogenicity of anti-DNA antibodies showed that double negative T cells (Lyt-1^{+}) were capable of inducing a cationic shift in the B cell generation of anti-DNA antibodies (Datta *et al.*, 1987).

CD4^{-}CD8^{-} double negative T cells produce only minimal Il-2 or Interferon-γ in response to mitogen stimulation in MRL/*lpr* mice (Davignon *et al.*, 1985). These cells do not appear to have significant cytotoxic activity, and thus this subset of T lymphocytes appears to have little potential for antigenic stimulation or lymphokine production. There appears to be a correlation between onset of disease and reduction in Il-2 production in MRL/++ mice (Altman *et al.*, 1981). In MRL/*lpr* mice this reduction is evident from an early age but does not manifest itself until other symptoms of disease, including B cell hyperactivity and autoantibody production, become apparent. In addition to a reduction in amount of Il-2 generated by these animals, analysis of T cells from lymph nodes and spleens of MRL/*lpr* mice showed that these cells failed to respond or responded only poorly to the mitogenic activator concanavilin A, even in the presence of exogenous Il-2 (Altman *et al.*, 1981).

1.3.7. The *lpr* gene/genetics

As MRL/++ mice develop a late-onset lupus-like syndrome, it can be concluded that the *lpr* gene defect accelerates rather than causes the disease. The *lpr* gene appears to be recessive, with *lpr*/++ heterozygotes developing a relatively mild disease, similar to that observed in MRL/++ homozygotes. However, there may be slightly increased mortality from lymphoid neoplasia. A marked reduction in Fas mRNA levels together with an abnormal restriction fragment pattern by Southern blot analysis detected by Watanabe-Fukunaga *et al.* (Watanabe-Fukunaga *et al.*, 1992a) showed that the *lpr* mutation is contained within the *Fas* gene. This gene, found on mouse chromosome 19 (Watanabe *et al.*, 1991), encodes a polypeptide which can mediate apoptosis. Fas is a 35kD protein with

an anchor localising it to the cytoplasmic face of the plasma membrane, expressed in both lymphoid and non-lymphoid tissues, with structural homology to the receptor for tumour necrosis factor, the low-affinity receptor for nerve growth factor and to the B-cell surface marker CD40 (Itoh *et al.*, 1991).

Additional DNA sequences from cDNA were amplified in *lpr/lpr* mice using primers that amplify sequences detecting a *Fas* deletion in genomic DNA, indicating that the additional sequences are probably intronic (Watson *et al.*, 1992). These researchers also detected a 1.4-kb deletion within the *Fas* gene by restriction fragment analysis and Southern blotting, and concluded that mRNA transcripts observed were probably non-functional. It has subsequently been shown that these aberrant transcripts arise from insertion of an early transposable element (ETn) into intron 2 of the *Fas* gene (Adachi *et al.*, 1993; Chu *et al.*, 1993) rather than an intragenic deletion. The Fas protein was detected on ~90% of Balb/c and MRL/++ thymocytes and was also found to be highly expressed on activated mature ($CD4^+$ or $CD8^+$) lymphocytes in those mice, but was absent in MRL/*lpr* mice (Drappa *et al.*, 1993). Consistent with these findings, activated MRL/*lpr* T cells have a defect in antigen-stimulated suicide, (Russell *et al.*, 1993) and replacement of the *lpr* gene with the normal *Fas* gene in T cells of transgenic MRL/*lpr* mice prevents accelerated autoimmune disease, eliminates generation of abnormal $CD4^-CD8^-B220^+$ cells and decreases autoantibody levels (Wu *et al.*, 1994).

The *lpr^cg^* gene is allelic to *lpr*, but complements the *gld* gene, i.e. *lpr^cg^*/++ and *gld*/++ heterozygotes develop lymphoproliferation. The *gld* gene is located on chromosome 1 (Roths *et al.*, 1984), and it was postulated that this gene encoded the Fas ligand (Cohen and Eisenberg, 1992). This appears to be the case, with *gld* disease caused by a point mutation in the carboxy-terminal region of the Fas ligand (Takahashi *et al.*, 1994). The *lpr^cg^* defect has been shown to be a single base pair substitution in the Fas antigen, resulting in replacement of an isoleucine with an asparagine residue and abolishing the ability to transmit

apoptotic signals (Watanabe-Fukunaga *et al.*, 1992b). Interestingly, *gld/lpr^cg* heterozygotes develop less severe lymphadenopathy than either homozygote (Matsuzawa *et al.*, 1990).

The presence of a homozygous *lpr* gene affects other strains of mice to varying degrees. Following transfer of the *lpr* gene there was a dramatic increase in mortality rate of two strains, C3H/HeJ and C57BL/6J, while the AKR/J strain of mice, which develops and dies of thymic lymphoma, showed little change. However, increased mortality was not due to renal failure, as *post-mortum* histological examinations revealed relatively benign renal lesions. All three strains demonstrated lymphadenopathy and increased concentrations of serum IgM and IgG, although the increases were somewhat limited in AKR/J mice. Interestingly, the range of antibodies produced by each strain differed, and there was evidence to suggest that background genes play a role in class switching from IgM to IgG anti-DNA antibody production in MRL/*lpr* mice. Certain SLE-specific features, such as the generation of anti-Sm antibodies, were not seen in *lpr* strains other than MRL/*lpr* (Izui *et al.*, 1984).

1.4: Hsps and SLE

1.4.1. Overexpression of hsps in SLE

The role of hsps in SLE remains unclear, although there is evidence to suggest that expression patterns of these proteins in at least a subset of patients with SLE is abnormal. In a preliminary study of pooled samples, Hsp90 and Hsp70 protein levels were shown to be elevated in the peripheral blood mononuclear cells (PBMCs) of 20 SLE patients compared with normal controls (Deguchi et al., 1987). A more thorough investigation in our laboratory of 57 patients with SLE revealed that only a proportion (~15%) had levels of Hsp90 in PBMCs that were regarded as elevated, i.e. exceeded the mean value plus three standard deviations of a control group of 12 healthy individuals (Norton et al., 1989). Overexpression of Hsp90 was not paralleled in a group of rheumatoid arthritis patients, and no correlation was observed between the levels of Hsp90 and steroid therapy or amounts of steroid used in treatment. Furthermore, all those patients with elevated protein levels were diagnosed as having active disease, although not all patients with active disease had elevated Hsp90 levels, and a serial study of Hsp90 levels of two patients with fluctuating disease symptoms appeared to show Hsp90 paralleling disease activity, with a concomitant decrease in Hsp90 levels as the disease flare waned.

This study was followed up by a more extensive survey of Hsp90 levels in PBMCs of 102 patients (222 blood samples) and 59 normal controls (102 blood samples). In addition, Hsp73, Hsp72 and Hsp60 levels were also determined, although smaller sample sizes were used to quantify those protein levels (Dhillon et al., 1993a; Dhillon et al., 1993b). Eighteen percent of SLE patients overexpressed Hsp90 protein compared with a control group, while 12% had elevated Hsp72 levels and one patient had increased amounts of Hsp73. There were no correlations between Hsp90 levels and Hsp72 or Hsp73 levels. Simple alterations

in the proportion of cell type were ruled out as a possible explanation for this overexpression, and highest levels were observed in patients with severely active disease, while no such correlation was observed in patients with high levels of Hsp72 protein. There were no correlations between Hsp90 or Hsp72 levels and anti-dsDNA antibody levels or Erythrocyte Sedimentary Rate (ESR), and patients taking prescribed drugs such as oral prednisolone, azathioprine or antimalarial drugs had no significantly different hsp levels compared with patients not taking those drugs.

The British Isles Lupus Assessment Group (BILAG) scoring system is a method for measurement of SLE disease activity which relies, at least in part, on patient input. The method defines eight categories of disease - General, Mucocutaneous, Neuropsychiatric, Musculoskeletal, Cardiorespiratory, Vascular, Renal and Haematological - and each category is scored on a five point scale, ranging from active (A) to never previously active (E) disease (Hay *et al.*, 1993). Using this system Hsp90 overexpression was linked to active disease in the neuropsychiatric and/or cardiorespiratory categories, whereas Hsp72 overexpression could not be categorised in this manner. Hsp90 levels in patients with active neuropsychiatric or cardiorespiratory disease also tended to parallel disease activity, clearly going from high to low or vice versa when either or both of these categories became inactive or active. In order to further categorise patients overexpressing Hsp90, a range of clinical parameters were examined to investigate possible links with Hsp90 levels (Dhillon *et al.*, 1994). The presence or absence of antibodies to Sm, RNP, Ro and La were independent of Hsp90 levels, but there appeared to be a link between high Hsp90 and antiphospholipid antibodies. In the same study HLA allotypes were investigated, and it was shown that Hsp90 levels were significantly higher in patients who were DR3-negative and A1/B8/DR3-negative.

Elevated levels of Hsp90 were investigated at a transcriptional level using nuclear run-on assays, and were shown to be dependent on enhanced

transcription of the Hsp90β, but not the Hsp90α gene (Twomey *et al.*, 1993). The transcription of these two genes were not correlated. The increased transcription of Hsp90β was not paralleled by enhanced transcription of the ubiquitin gene (Twomey *et al.*, 1992). Furthermore, although 10% of SLE patients had elevated Hsp70 gene transcription, no correlation was observed between gene transcription and protein levels (Twomey *et al.*, 1993). A small study of five patients by Deguchi and Kishimoto (Deguchi and Kishimoto, 1990) also noted an increase in Hsp70 gene transcription, but protein levels were not measured for comparison. Different gene regulatory mechanisms therefore exist to account for overexpression of Hsp70 and Hsp90 in SLE. Hsp70 overexpression must involve either increased stability of the protein or increased translation of mRNA into protein.

The role of Hsp90 in SLE was examined further by a study which was undertaken to investigate surface expression of the protein in PBMCs (Erkeller-Yüksel *et al.*, 1992). In a sample of 62 patients, 20% had surface Hsp90 levels above the mean + 2SD of controls. PBMCs were subdivided into lymphocytes and monocytes and similar overexpression patterns were observed in both cell classes, although this was not statistically significant in the monocyte population. The lymphocyte population was further characterised, and surface expression of Hsp90 was shown to be linked to the B lymphocyte population and a small proportion of CD4$^+$ T cells. Disease activity of patients was assessed and elevated surface Hsp90 expression was seen in 8/35 patients with active disease compared with 1/18 patients with inactive disease. There was no correlation of surface Hsp90 levels with ESR, absolute lymphocyte counts, dsDNA levels or C3 measured on the same day. However, surface expression of Hsp90 was linked with activity within the musculoskeletal group mentioned above.

1.4.2. Antibodies to hsps

The first hints that large self hsps might be recognised by the immune system in SLE patients was seen in a study which revealed the presence of IgG autoantibodies to Hsp90 in sera of ~50% of patients, but not in sera from healthy controls or patients with rheumatoid arthritis or scleroderma (Minota *et al.*, 1988a). This study was followed by a parallel investigation of IgG and IgM autoantibodies to the constitutive Hsp70 protein, Hsp73 (Minota *et al.*, 1988b). IgM antibodies were found in 13/34 adult patients, while 5/34 patients had IgG antibodies to Hsp73. No autoantibodies to Hsp73 were found in 10 normal sera, and were observed infrequently in other disease sera. These results have been challenged by the findings of another group, who discovered similar frequencies of autoantibodies to both Hsp73 and Hsp72 in SLE patients and healthy subjects (Kindås-Mügge *et al.*, 1993). Several reasons were offered for the discrepancy between the two sets of results, including assay sensitivity, different antigenicity between the proteins used as antigens in the experiments, and different incubation times, which may have allowed greater time for low affinity antibodies to bind. These authors also failed to uncover any correlations between presence of anti-Hsp70 autoantibodies and clinical or serological manifestations of SLE disease.

An extensive study of autoantibodies to Hsp90 and Hsp70 in SLE patients has been conducted in our laboratory using an ELISA system to detect autoantibodies to the hsps (Conroy *et al.*, 1994). Seventy-two SLE patients were examined, as well as patients with myositis, rheumatoid arthritis, Sjögren's syndrome, scleroderma and osteoarthritis. Elevated levels of IgG autoantibodies to Hsp90 were detected in 26% of patients with SLE, while IgM autoantibodies were seen in 35% of the same sample of patients. Similar frequencies were found in myositis, but only 11% of the Sjögren's syndrome patients and 4% of healthy controls had elevated anti-Hsp90 IgG levels, and these autoantibodies were

absent in patients with rheumatoid arthritis, scleroderma or osteoarthritis. Likewise, elevated anti-Hsp90 IgM levels were found in a small proportion of patients with scleroderma (13%) and rheumatoid arthritis (7%), as well as healthy controls (4%), but not in patients with osteoarthritis or Sjögren's syndrome. As with the study by Kindås-Mügge et al. mentioned above (Kindås-Mügge et al., 1993), similar frequencies and levels of autoantibodies to Hsp70 were seen in all groups examined.

Elevated anti-Hsp90 levels were not correlated with protein levels measured on the same day or on a previous occasion. Anti-Hsp90 IgM levels were correlated with active renal disease, and elevated antibody levels (IgM and IgG combined) also matched with a low C3 level. No correlation between anti-Hsp90 and anti-DNA levels were observed, nor were there any correlations between anti-Hsp90 levels and steroid therapy or level of steroid administered. A genetic association was discovered between patients with raised antibody levels and who were HLA type A1/B8/DR3-negative, a link mirrored in patients with elevated Hsp90 protein levels (Dhillon, 1993). A recent study investigating the role of Hsp90 in a range of fungal diseases as well as SLE revealed differences in epitopes of the protein being recognised by patients with different diseases (Al-Dughaym et al., 1994). All six SLE patients tested had antibodies to a 47kD breakdown component of Hsp90, and 5/6 SLE patients had antibodies to the entire Hsp90 protein, although the mean antibody levels in patients with SLE was lower than levels in patients with infectious diseases. SLE patients responded to 8/12 of the epitopes tested, with 5/6 patients recognising an epitope corresponding to the sequence KNDK in the human Hsp90 protein and 4/6 patients recognising epitopes KIRY, GLELPE and LDKK.

Antibodies to ubiquitin have been discovered in a high proportion of SLE patients (Muller et al., 1988). In this survey, 79% of patients had antibodies to this protein, much higher than patients with rheumatoid arthritis (15%), scleroderma (16%), healthy controls (3%), or SLE patients with a positive anti-

DNA test (55%). This group also showed that p52 and p43 complexes containing ubiquitin and histones H2A and H2B were strongly recognised by autoantibodies.

1.4.3. Murine models

Little work has been undertaken into the role of hsps in murine models of SLE. However, one study has investigated levels of Hsp70 transcription in kidney lymphoid cells from MRL/*lpr* mice and compared them to levels from MRL/++ mice (Deguchi, 1991). An increased amount of Hsp70 RNA was observed in MRL/*lpr* mice, resulting from increased transcription of the hsp gene. There was good correlation between enhancement of Hsp70 transcription and deterioration of lupus-related renal disorders, suggesting a potential role in development of the disease state.

1.4.4. Aims of this thesis

The discovery of elevated Hsp90 and Hsp70 protein levels in patients with SLE has raised many questions regarding the relevance of this elevation to pathogenesis of disease. The mortality rate from SLE has decreased somewhat over the past couple of decades with the introduction of steroid and other new therapies, and there are obvious difficulties in obtaining tissue samples from internal organs of patients. Furthermore, since nothing is known about the aetiology of SLE, there is no possibility of obtaining samples from patients prior to onset of disease. Hence, there are limitations to studies that can be performed on human patients, and murine models have been utilised in an attempt to understand the nature of lupus-like illnesses.

Animal models are beneficial for a variety of reasons:

(1) In murine lupus models such as MRL/*lpr* and NZB/W the disease is predictable, following a defined course, and it is thus possible to conduct temporal studies and to examine mice prior to disease onset.

(2) Tissues and organ systems can be more readily and thoroughly studied, enabling researchers to focus on sites of disease activity.

(3) Experimental supplies are limited only by the size of any given colony.

(4) Novel therapies can be devised and tested on these animals before proceeding to clinical trials.

(5) Results of experiments are less complicated. Human patients are inevitably undergoing treatment, and these treatments may have a bearing on disease parameters under investigation.

(6) Use of inbred strains enables researchers to avoid the complexities of heterogeneity.

Although the cause of accelerated disease in MRL/*lpr* mice has now been elucidated, namely an insertion of a retrotransposon into the *lpr* gene (Watanabe-Fukunaga *et al.*, 1992a; Chu *et al.*, 1993) much work remains to be done to fully understand the nature of the disease in this strain. Many features of this disease parallel those in human SLE patients, although it is clear that no such defect in the human gene encoding the Fas antigen exists which could account for the wide range of symptoms documented in the human disease. However, there may be common mechanisms underlying similar features of the two diseases, and the MRL/*lpr* mouse remains a valuable experimental tool for investigation of SLE.

This project was instigated to extend our previous investigations into the role of Hsps in SLE by examining MRL/*lpr* mice, initially through confirmation that a comparable situation exists in the MRL/*lpr* strain with regard to overexpression of hsps. The success of that investigation would thus lead into a project

examining the role of hsps in pathogenesis of MRL/*lpr* disease. By utilising the animal model it would be possible to investigate the relationship between hsp levels and the onset and course of disease. It was also appropriate to search for an antibody response to hsps in MRL/*lpr* mice, as the theory of molecular mimicry as a potential cause of autoimmune disease relies on a candidate protein being accessible to the immune system. A breakdown in tolerance would consequently lead to targeting of these molecules by the immune system, and hence an autoantibody response.

CHAPTER 2: MATERIALS AND METHODS

2.1: Materials

Tissue samples

Balb/c, MRL/*lpr* and MRL/++ mice were obtained from colonies maintained in the University College and Middlesex School of Medicine animal house. Additional animals were obtained from Harlan Olac Ltd., Bicester, England, who also supplied the original stocks for generating the colonies.

Plasmids

The following plasmids were used in nuclear run-assays:

Plasmid	Derived from	Plasmid	Derived from
PJ3	Hsp90	2-3	Hsp70
90.5	Hsp90	p17	Hsp70
Hsp α	Hsp90	hsc70	Hsp70
90.3	Hsp90	DP8	Hsp70
SmB	SmB	pH8	Hsp27
PP1	SmN	pλ22A	Hsp60
201	SmB	123	Ribosomes

Table 2.1. Plasmids used in nuclear run-on assays. Plasmid 123 was used as a positive control in this assay and to correct for variation between samples.

Chemicals

Unless otherwise stated all alcohols were obtained from BDH, Lutterworth, Leicestershire. All solid chemicals were dissolved in water, adjusted to the correct pH with HCl or NaOH, autoclaved or filter-sterilised and stored at room

temperature unless otherwise stated. SDS [BDH] and NaOH [BDH] were not autoclaved, while phenol [BDH], phenol/chloroform, chloroform [BDH], and ethidium bromide [Sigma Immunochemicals, Poole, Dorset] were stored in the dark. Chloroform always contained 4% v/v isoamyl alcohol [Sigma], phenol contained 0.1% w/v hydroxyquinoline [Sigma] and was buffered by shaking three times with an equal volume of 0.5M Tris [BDH] pH 8.0, and with 0.1M Tris pH 8.0, 0.2% β-mercaptoethanol [BDH] twice, removing the aqueous layer each time. Formamide [BDH] was deionised by stirring with 10% w/v duolite MB 6113 mixed resin [BDH] for 30 minutes and subsequently filtering through 3MM paper. Solutions treated with 0.1% v/v diethylpyrocarbonate (DEPC - [Sigma]) were shaken for at least 3 hours and autoclaved. DNAase-free RNAase-A [Boehringer Mannheim, Lewes, East Sussex] was prepared by boiling for 10 minutes at 10mg/ml in dH_2O and was stored at -20°C.

General equipment

All plasticware for ELISAs was obtained from Life Technologies, Paisley and all other disposable plasticware was obtained from Greiner Labortechnik Ltd., Dursley, Gloucester. Film fixer and developer was obtained from Photosol, Genetic Research Instrumentation, Little Dunmow, Essex. Autoradiography was carried out at -70°C with an intensifying screen. 3MM chromatography paper used in Western blotting and nuclear run-on assays was obtained from Whatman, Maidstone. Radiolabelled cytosine triphosphates - [α-^{32}P]-dCTP (10μCi/μl 3000Ci/mmol) and [α-^{32}P]-CTP (10μCi/μl 800 Ci/mmol) were obtained from DuPont, Stevenage, Hertfordshire. Cell lines were obtained from the Imperial Cancer Research Fund, Lincoln's Inn Fields, London.

Statistical analyses were undertaken on a Macintosh LCII computer using the statistical software package Exstatix, version 1.0.3 [Select Micro Systems, New York, USA] or the statistical functions of Cricket Graph III, version 1.0 [Computer Associates International Inc., New York, USA].

2.2: Methods

2.2.1. Western blotting

(i) Preparation of protein samples

Mice were killed under anaesthetic by cardiac puncture, dissected and the desired whole organs removed, snap frozen in liquid N_2 and stored at -70°C until required. Protein samples were prepared by cutting and weighing the whole tissue and homogenising with an Ultra-turrax T25 homogeniser [Greiner] in gel loading buffer (appendix 1) at a concentration of 100mg tissue/1ml gel loading buffer. Samples were sonicated in a sonicating water bath for 20 minutes, boiled for 5 minutes and stored at -20°C until required. The samples were reboiled for 5 minutes immediately prior to loading on an acrylamide gel.

(ii) Acrylamide gels

Protein samples were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (Laemmli, 1970).

Resolving gels were diluted to a final concentration of 10% acrylamide, 0.25% bis-acrylamide, from a stock solution of 30% acrylamide, 0.8% bis-acrylamide [National Diagnostics, Atlanta, Georgia, USA]. The gels comprised 1.5M Tris, 0.4% SDS, pH8.8 and were polymerised with 0.01% N'N'N'N' tetramethylethylene diamine (TEMED-[Sigma]) and 0.01% ammonium persulphate [BDH]. The stacking gels comprised 0.5M Tris, 0.4% SDS, pH6.8, and were similarly polymerised with TEMED and ammonium persulphate.

Gels were run at a constant 50mA for 2-3 hours in running buffer (appendix 1). Mid-range molecular weight protein standards (Rainbow markers, [Amersham, Little Chalfont, Buckinghamshire]) or standard molecular weight markers [Life Technologies] were run with each gel. Samples were run in duplicate, with half of the gel stained for protein and the other half used for Western blotting.

(iii) Coomassie Staining

Each half of the gel required for non-specific protein staining was placed in a solution containing Coomassie Blue R [Sigma], 50% methanol [BDH] and 7% acetic acid [BDH]. This was placed on a rocking platform at room temperature overnight. The following day any unbound stain was washed off in a 50% methanol, 10% acetic acid destaining solution. Gels were either wrapped in plastic wrap and placed at 4°C or dried using a model 583 gel dryer [Bio-Rad, Hemel Hempstead, Hertfordshire] and stored until required.

(iv) Blotting

To probe for specific proteins relevant gels were blotted overnight onto Hybond C nitrocellulose [Amersham] in blotting buffer (appendix 1) at 210mA. Following blotting the unfilled binding sites on the nitrocellulose were blocked by placing the nitrocellulose in a solution containing 10% dried skimmed milk powder [Tesco Ltd., Cheshunt, Middlesex], 0.2% polyoxyethylenesorbitan monolaurate (Tween20 - [Sigma]) in Tris-buffered saline (TBS - appendix 1) for at least one hour at room temperature.

The following antibodies were used:

(i) Ac88, a monoclonal antibody against Hsp90, is a yeast-derived antibody (a kind gift of Dr. David Toft., Mayo Clinic, Rochester, USA). This antibody was developed against Hsp90 isolated from the water mould *Achyla ambisexualis* (Riehl *et al.*, 1985). Ac88 recognises several small proteins in the 30-40kDa range as well as Hsp90, and these proteins have been identified as heterologous nuclear RNPs (Minoo *et al.*, 1989). Reactivity with these proteins makes Ac88 unsuitable for use in development of an enzyme-linked immunosorbent assay (ELISA) assay. For Western blotting experiments Ac88 was used at a final concentration of 10μg/ml in washing buffer (5% milk powder, 0.2% Tween20 in TBS).

(ii) SPA-801 (Stressgen Biotechnologies Corporation, Sidney, Canada) is an antibody that recognises Hsp72, the heat inducible member of the Hsp70 family. SPA-820, a mouse monoclonal antibody which recognises both Hsp72 and the constitutive Hsp73 protein was used to determine levels of Hsp73.

(iii) Mouse monoclonal antibody 4B9/89 was a gift from Dr. Graham Rook, Department of Medical Microbiology, University College and Middlesex School of Medicine, University College, London. It is a monoclonal antibody that recognises the human form of the chaperonin protein Hsp60, but not the bacterial homologue GroEL.

After blocking with milk protein the nitrocellulose strip was washed (1 x 5 minute wash) in washing buffer and incubated on a rocking platform at room temperature for two hours with the relevant antibody at the concentrations noted above. Unbound antibody was rinsed off in washing buffer (3 x 5 minutes), and the blot was incubated for one hour with Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins [Dako, High Wycombe], diluted 1:1000 in washing buffer. The blot was rinsed (3 x 5 minutes washing buffer, 1 x 10 minute TBS), and probed for the protein using enhanced chemiluminescence (ECL™).

(v) Detection of antibodies to Hsp90

Pooled sera from MRL/*lpr* mice was used to determine whether antibodies detected in an ELISA could be visualised using a Western blotting system. Purified Hsp90 was subjected to SDS-PAGE and blotted as above. For the detection of antibodies pooled sera from 10 anti-Hsp90-positive and 10 anti-Hsp90-negative MRL/*lpr* were used at a concentration of 1:20 in washing buffer and the procedure followed as described with longer blocking (at least 4 hours) and washing (3 x 15 minutes) periods to minimise non-specific binding of serum proteins.

(vi) Enhanced Chemiluminescence

Peroxidase and hydrogen peroxide can be utilised to catalyse the oxidation of luminol in alkaline conditions. This reaction leaves oxidised luminol in an excited state which then decays to a ground state, releasing light in doing so (Fig.2.2.1). The enhanced chemiluminescence technique uses phenolic enhancers to increase and prolong output of light from this reaction. The light output from this reaction has a maximum light emission wavelength suitable for detection on autoradiographic film. Blots were placed in a mixture of the two solutions provided and rinsed for precisely one minute. The blots were drained thoroughly, wrapped in plastic wrap and exposed to either XAR fast film [Kodak, Hemel Hempstead, Hertfordshire] or Hyperfilm-ECL [Amersham] film.
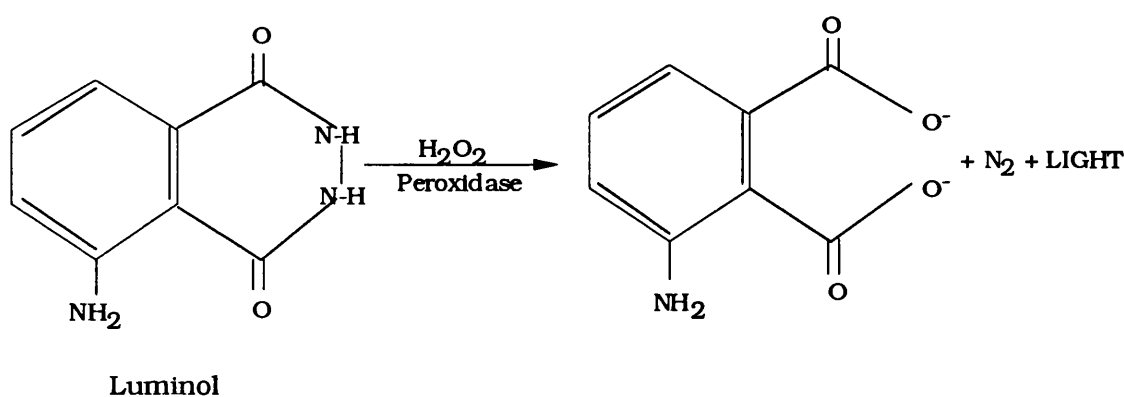
Luminol

Fig.2.2.1. Luminol releases light in the presence of hydrogen peroxide and Horseradish peroxidase. This reaction forms the basis for chemiluminescent detection of peroxidase conjugated proteins.

(vii) Quantitation of protein bands

Developed films or dried gels were scanned using a VD620 scanning densitometer [Bio-Rad] or a GS-620 imaging densitometer [Bio-Rad] within the linear range, and analysed using Windows Image Analysis software [Microsoft,

California, USA]. The values for the Hsp90 band (H) from one half of an SDS-PAGE gel were divided by the values obtained from the actin band (A) from the destained Coomassie Blue-stained gel, i.e. H/A. These values were equalised for inter-gel differences using the H/A value from a standard. Protein samples from the Jurkat T cell line (see section 2.2.5) were used as standards for these experiments, and were batch-tested and calibrated between successive generations to ensure maximum reproducibility. The calculation for determination of the hsp value of an unknown sample thus becomes: H/A (sample) - H/A (Jurkat) x E, where E is the equalisation factor.

2.2.2. Flow cytometry

(i) Preparation of cells for flow cytometry

To prepare cells for FACS analysis whole spleens were dissected from freshly-sacrificed mice, placed in wash buffer (2% Fetal Calf Serum [Life Technologies], 0.1% sodium azide [BDH] in phosphate-buffered saline, PBS) and single cell suspensions made by teasing the tissue apart with forceps. To isolate the lymphocytic population this suspension was spun through a Ficoll gradient [Pharmacia, Milton Keynes] at 9000r.p.m. in a Centra-4R centrifuge [Damon, Dunstable, Bedfordshire] for 25 minutes.

For labelling to use in flow cytometry cells were spun at 400g for five minutes at 4°C, washed in wash buffer and respun. Antibody, diluted appropriately (as below) in washing buffer, was added to the cell pellets and the cells resuspended and left on ice for one hour. 500μl of wash buffer was added to the suspension and the preparation was washed and spun twice. This sequence was repeated for each antibody before final resuspension of cells in ~250μl wash buffer. To test for viable cells 50μg/ml propidium iodide (PI - [Sigma]) in PBS (appendix 1) was added prior to FACS analysis.

(ii) Surface staining

Cells were stained with the desired fluorochromes individually and together (1 x 10^7 cells for multiple stains; 7.5 x10^6 cells for single stains). For the detection of B cells phycoerythrin-labelled anti-mouse total immunoglobulin [Southern Biotechnologies, Birmingham, Alabama, USA] was used at a final dilution of 1:20 in wash buffer, while surface expression of Hsp90 was examined using fluorescein isothiocyanate (FITC - [Sigma])-labelled Ac88 (see below), diluted in wash buffer 1:20.

(iv) FITC-labelling of antibodies for flow cytometry

Purified monoclonal antibody to Hsp90 (Ac88) was dialysed against FITC labelling buffer (appendix 1) at 4°C with three changes over 48 hours. The antibody concentration was determined based upon spectrophotometry at OD_{260} and 20µl FITC (5µg/ml) added for each milligram of antibody. The preparation was incubated for a minimum of two hours at room temperature and dialysed against TE to remove unbound FITC against dialysis buffer (appendix 1) at 4°C over two days. FITC-labelled Ac88 was stored in the dark at 4°C until required.

(v) FACS analysis

FACS analysis was performed using Becton-Dickenson FACScan apparatus [Becton-Dickenson, Oxford] and recommended protocols. Unstained cells were passed through the machine and used to adjust gains and voltages for investigation of surface staining. Following standardisation stained cells were passed through the FACScan at a flow rate of approximately 500 cells/second and stored on computer diskette at 10 000 events/sample. Gates were set after addition of PI to exclude non-viable cells.

2.2.3. Nuclear run-on assay

(i) Preparation of nuclei from tissues

Tissues were taken from freshly killed mice, weighed and minced in 2 volumes 0.25M sucrose in TKM (appendix 1). 500µl of these preparations was mixed with 500µl 2.3M sucrose/TKM. The tubes were underlain with 500µl 2.3M sucrose/TKM and spun at 32 000r.p.m. in a Beckman TL-100 ultracentrifuge [Beckman, High Wycombe, Buckinghamshire] for 30 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 20-40µl DEPC-treated TKM. Two volumes NP40 lysis buffer (appendix 1) were added and the solution left on ice for 10 minutes. The preparation was underlain with 100µl 2.3M sucrose/TKM and centrifuged at 10 000g for 10 minutes at 4°C. (Cell lysis was repeated until the nuclei appeared as a clean white pellet). The pellets were resuspended in 100µl freezing buffer (appendix 1) and stored at -70°C until required.

(ii) Preparation of nuclei from cell lines

Cells were washed in PBS and spun at 500g in a microfuge at 4°C. The pellet was resuspended in 500µl NP40 lysis buffer and the mixture left on ice for 10 minutes before underlying with lysis buffer/30% sucrose and spinning at 500g for 3 minutes at 4°C. The pellets were resuspended in 100µl freezing buffer and stored at -70°C until required.

(iii) Plasmid amplification and purification I

Ten microlitres of a glycerol stock of the required plasmid-containing bacteria was used to inoculate 5ml L-broth (appendix 1). The culture was grown overnight in a 37°C shaking orbital incubator and used the following day to inoculate 500ml L-broth in a 2-litre glass flask. The cultures were grown

overnight in a shaking incubator at 37°C. and centrifuged at 7000r.p.m. for 10 minutes at 4°C in a Sorvall J2-21 centrifuge [Dupont], using a JA-10 rotor.

The cell pellet was resuspended in 4ml solution 1 (appendix 1) and left on ice for 15 minutes before EDTA was added to a final concentration of 10mM from a 0.2M stock. This was left on ice for 15 minutes.

0.5 volumes Triton buffer (appendix 1) was added and after thorough mixing the preparation was left on ice for a further 30 minutes. The culture was spun in a J2-21 centrifuge, using a JA-20 rotor for one hour at 18 000r.p.m. and the supernatant transferred to a 50ml Falcon tube [Becton Dickenson].

Tris-HCL, pH7.5 and NaCl [BDH] were added to concentrations of 50mM and 0.5M respectively, and to this mixture an equal volume of 1:1 phenol/chloroform was added. Tubes were spun at 4000r.p.m. in a Centra 4-R centrifuge for 10 minutes and the upper phase transferred to a fresh Falcon tube.

An equal volume of chloroform was added and the tubes were vortexed and respun in the Centra 4-R. The upper phase was again transferred to a clean Falcon tube. Polyethylene glycol (PEG) 6000 [BDH] was added (10% w/v) and dissolved in a 37°C shaking incubator before storing overnight at 4°C.

The following day the tubes were spun at 12 000r.p.m. in a J2-21 centrifuge for 20 minutes at 4°C. The pellets were resuspended in 500μl 0.1M Tris, pH8, and 10μl RNAase A (10mg/ml), transferred to 1.5ml Treff tubes [Scotlab, Strathclyde] and left at 37°C for 30 minutes. An equal volume of solution 2 (appendix 1) was added and left on ice for one hour before spinning at 5000r.p.m. for 15 minutes. The pellet was resuspended in 400μl 10mM Tris (pH8), 0.5M NaCl, and an equal volume of phenol was added to each preparation. The tubes were spun in the microfuge for 5 minutes and the upper phase transferred to a clean tube. An equal volume of chloroform was added and the tubes spun again in the microfuge for 5 minutes, the upper phase recovered, and DNA precipitated by addition of 1/10 volume 4M NaCl and 2 volumes absolute ethanol. This was stored overnight in a -70°C freezer.

The preparations were spun for 10 minutes in the microfuge, the supernatant discarded and 750µl 70% ethanol added to each Treff tube before recentrifugation in the microfuge for 5 minutes. The pellet was dried in a vacuum freeze dryer or in air and resuspended in 500µl sterile dH_2O. The concentration of DNA was determined by spectrophotometry at OD_{260}.

(v) Plasmid amplification and purification II

Plasmid DNA was also isolated using the Promega Wizard Maxiprep DNA Purification System [Promega, Southampton]. Two-litre conical flasks containing 500ml L-broth were inoculated with 10ml overnight cultures of an appropriate plasmid-containing bacterial stock and grown overnight in a shaking 37°C incubator. The culture was centrifuged at 14 000g for 10 minutes at 4°C and the pellet resuspended in 15ml cell resuspension solution (appendix 1). Once the lysate had become clear an equal volume of 1.32M potassium acetate, pH4.8, was added and the resultant mix centrifuged at 14 000g for 15 minutes at 4°C. The supernatant was transferred to a fresh tube and 0.6 volumes isopropanol added. The solution was centrifuged at 14 000g for 15 minutes at 4°C and the pellet resuspended in 2ml TE buffer, pH7.5 (appendix 1). The suspension was passed through the prepacked columns as per the recommended protocol, and the plasmid DNA stored in dH_2O at -20°C.

To purify the preparations 10µl of 10mg/ml RNAase A was added and the mixture incubated at 37°C for at least 30 minutes. Phenol was added in a 1:1 ratio and the solution spun in a microfuge for five minutes. The upper phase was recovered, 400µl chloroform was added and the preparations were spun as above. The upper phase was taken and the DNA precipitated with 1/10 volume 4M NaCl, 2 volumes absolute ethanol at -70°C overnight.

The following day the solution was spun in the microfuge, washed in 750µl 70% ethanol and respun. The pellet was freeze-dried and resuspended in 500µl dH_2O and the concentration of DNA determined by spectrophotometry at OD_{260}.

The purity and integrity of plasmids obtained by both methods was always checked by cutting plasmids with appropriate restriction enzymes and visualising plasmids on a 1% agarose gel (appendix 1), typically run at 100mA in 1 x TBE (appendix 1). Plasmids were visualised on a UVP transilluminator [UVP Products, Cambridge, UK]. Typical yields were 400-1000μg DNA using method (i) and 250-500μg DNA using method (ii).

(vi) Preparation of filters

For each spot of plasmid, 5-10μg of DNA was boiled in 0.3M NaOH for 5-10 minutes and allowed to cool. Ammonium acetate, pH5.5, was added to a final concentration of 2M and an aliquot of Bromophenol blue [Sigma] in dH_2O added to facilitate visualisation. Samples were spotted onto a Hybond-N [Amersham] nylon filter that had been pre-soaked in 2 X SSC, using a Bio-Dot SF slot blotting apparatus [Bio-Rad]. The filters were baked at 180°C for 2 hours or cross-linked in a UV Stratalinker [Stratagene, Cambridge, UK]. For prehybridisation, each filter was added to a bag containing 5ml nuclear run-on prehybridisation buffer (appendix 1); the bags were sealed and placed in a 42°C Hybaid oven [Hybaid Ltd., Teddington, Middlesex] overnight before the addition of probe.

(vii) Nuclear run-on assay

A nuclear run-on reaction mix (appendix 1) was mixed with $\alpha-^{32}P$-labelled CTP, added to each preparation of nuclei and incubated for one hour at room temperature. A solution containing 1μl VRC [Life Technologies], 0.4μl tRNA [Boehringer Mannheim] (100mg/ml standard) and 0.5μl RNAase-free DNAase [Boehringer Mannheim] was added to each preparation and left to incubate at room temperature for 30 minutes. To this was added 2μl 1M Tris pH7.5, 5μl 250mM EDTA, 20μl 10% SDS and 4μl 10mg/ml Proteinase K [Boehringer

Mannheim]. The Proteinase K was pre-treated at 37°C for 30 minutes to destroy RNAases.

The preparation was incubated for one hour in a 37°C water bath, 200µl phenol/chloroform was added, and the solutions were mixed and centrifuged in a microfuge for 5 minutes. The upper phase was retained, added to 600µl ice-cold RNAase-free ethanol and stored overnight at -20°C.

The following day the tubes were spun for 10 minutes in a microfuge and the pellets washed in 70% ethanol, respun and resuspended in 100µl RNAase-free dH_2O. These were added to pre-hybridised filters and left to hybridise in a 42°C Hybaid oven for 3-4 days.

Following hybridisation the filters were washed in 2x SSC, 0.1% SDS at room temperature, with a further wash in the same solution at 65°C if the filters were still hot. If necessary, a further wash was conducted in 2x SSC, with the addition of 20µg/ml RNAase A at room temperature for 30 minutes. The filters were exposed to film overnight.

2.2.4. ELISAs

(i) Preparation of sera samples

Mouse blood was collected from Balb/c, MRL/*lpr* and MRL/++ mice by retro-orbital eye bleed. The blood was allowed to clot at room temperature for at least one hour and the sera extracted by centrifugation in a Microfuge at 6500r.p.m. for 10 minutes. The sera were dispensed into 5µl aliquots and stored at -20°C until required.

(ii) Hsp90 assay

Ninety-six well Nunc Maxisorp flat-bottomed ELISA plates [ICN Flow, California, USA] were coated overnight with 100µl purified human Hsp90 [Stressgen, > 90% purity] in bicarbonate buffer (BIC) pH9.6 (appendix 1). The protein was added at

86

three different concentrations to each plate - 2µg/ml, 0.5µg/ml and 0.1µg/ml - with a BIC control. Plates were incubated overnight at 4°C.

The following day plates were washed in BIC and unfilled binding sites on the ELISA plates blocked with 100µl 1% Bovine Serum Albumin (BSA) in BIC for 1hr at 37°C.

Plates were washed 6x in PBS/0.05% Tween20 (PBS-T) using a Dynawasher II [Dynatech, Billinghurst, West Sussex], and 100µl samples added. Samples were prepared by diluting neat sera 1:200 in 1% BSA/1% goat serum [Seralab, Crawley Down, UK]/PBS-T and leaving to stand at room temperature for at least 60 minutes prior to the plates being coated. The BSA/BIC and BSA/goat serum/PBS-T were filtered prior to use through a 0.22mm filter [Sartorius, Epsom].

Plates were incubated for 1hr at 37°C, washed 6x with PBS-T and 100µl alkaline phosphatase-conjugated goat anti-mouse F_C-specific IgG [Sigma] diluted 1:1000 in BIC added. Plates were left at 4°C overnight.

The following day the ELISA plates were washed and developed using 1mg/ml dinitrophenyl phosphate (substrate) tablets [Sigma], 0.002M MgCl [BDH]. Results were obtained using a Dynatech MR4000 ELISA reader [Dynatech] at OD_{405}, reference OD_{490}. Results were equalised for inter-plate-variation by generating a standard curve from a positive control serum on each plate, while intra-plate variation was regularly monitored by coating a plate with a single serum under the same conditions. No variation greater than 10% was observed in these control experiments. Ac16, a monoclonal antibody that recognises the native form of the Hsp90 protein, was used as the positive control for the anti-Hsp90 assay at a concentration of 1µg/ml. The antibody was a kind gift from Dr David Toft.

(iii) Hsp90 IgM assay

The procedure followed was as above, using 100µl alkaline phosphatase-conjugated goat anti-mouse IgM [Sigma] instead of the IgG second layer.

(iv) Hsp70 and Hsp60 assays

The method followed was as above, replacing Hsp90 with purified bovine Hsp70 [Stressgen] or purified full-length recombinant Hsp60 (a gift from Dr R. Gupta). Purified Hsp70 (> 95% purity) is a mixture of the human 72kD and 73kD proteins (Hsp70 and Hsc70 respectively), the predominant form being the constitutive Hsp73 protein. The Hsp60 gave a single band when run on an SDS-PAGE gel.

The positive controls used for these assays were SPA-801, an anti-Hsp72 antibody [Stressgen], and the monoclonal antibody 4B9/89, both at a concentration of 0.5mg/ml.

(v) Anti-DNA ELISA

Maxisorp plates were coated with 100μl poly-L-lysine [Sigma] at a final concentration of 50mg/ml in dH_2O and incubated for 1hr at 37°C, then washed with dH_2O.

Double and single-stranded DNA, at concentrations of 5mg/ml and 10mg/ml respectively, were used to coat the plates, with a BIC control. Double-stranded DNA [Sigma] was prepared by incubation with 1:10 S$_1$ nuclease [Sigma] at 37°C for 60 minutes.

The ELISA plates were incubated overnight at 4°C, washed 3x in PBS and blocked with 100μl of a 100mg/ml poly-L-glutamate solution [Sigma] at 37°C for 1hr. The plates were washed 3x with PBS, blocked with 100μl 2% BSA/BIC at 37°C for 1hr and washed 5x with PBS-T using the Dynawasher.

Serum samples were added as for the Hsp90 assay above and the plates incubated for an hour at 37°C before a further set of PBS-T washes with the Dynawasher.

The samples were labelled with 100μl mixed alkaline phosphatase-conjugated goat anti-mouse antibodies [Sigma]. These were prepared using 1:1000 dilutions of anti-IgG, anti-IgM and anti-IgA antibodies in 1% goat serum/PBS-T. The plates were incubated at 37°C for 1hr and washed 6x with PBS-T in the Dynawasher.

Substrate was added as for the Hsp90 assay and the results of colour development scanned at OD_{405} (ref. OD_{490}) using the Dynatech reader.

(vi) Total IgG assay

Maxisorp plates were coated overnight at 4°C with 5mg/ml anti-mouse IgG [Sigma] in BIC. Plates were rinsed twice with PBS, blocked for one hour at 37°C with 2% BSA/BIC, and rinsed again with PBS. A standard curve was constructed by adding serial dilutions of mouse IgG [Sigma] in 1% BSA/BIC from 40mg/ml - 6.25 x 10^{-2} mg/ml. Mouse sera was diluted serially 1:2000 - 1:16 000 in the same buffer and incubated at 37°C for one hour, followed by extensive washing in PBS-T. Alkaline phosphatase-conjugated anti-mouse IgG [Sigma] was added at a dilution of 1:5000 and incubated at 37°C for one hour. The plates were washed in PBS-T and rinsed in BIC, substrate was added and colour developed and read in the Dynatech reader as per the protocols above.

2.2.5. Hsp90 promoter

(i) Hsp90 promoter constructs

Four plasmid constructs of the human Hsp90 promoter were used for the investigation of promoter control mechanisms in cell lines. The promoter constructs were made in the laboratory of Dr N. Rebbe, and were a kind gift to our group. They contain segments of the Hsp90β gene and promoter, replacing the Hsp90 protein coding segment with the chloramphenicol acetyltransferase (CAT) reporter gene (Rebbe *et al.*, 1989). Fig.2.2.2. shows a schematic drawing of the four constructs. Construct A includes three consensus heat shock elements (HSEs) at the 5' end of the construct, but has no intronic HSEs. This contrasts with construct D, which has one complete and one partial consensus HSE sequence within the intronic sequence of the gene. Construct B is the full promoter, containing the known HSEs, and construct C is the negative control,

Plasmid constructs of the human Hsp90 promoter

Hsp90 gene

Construct A

Construct B

Construct C
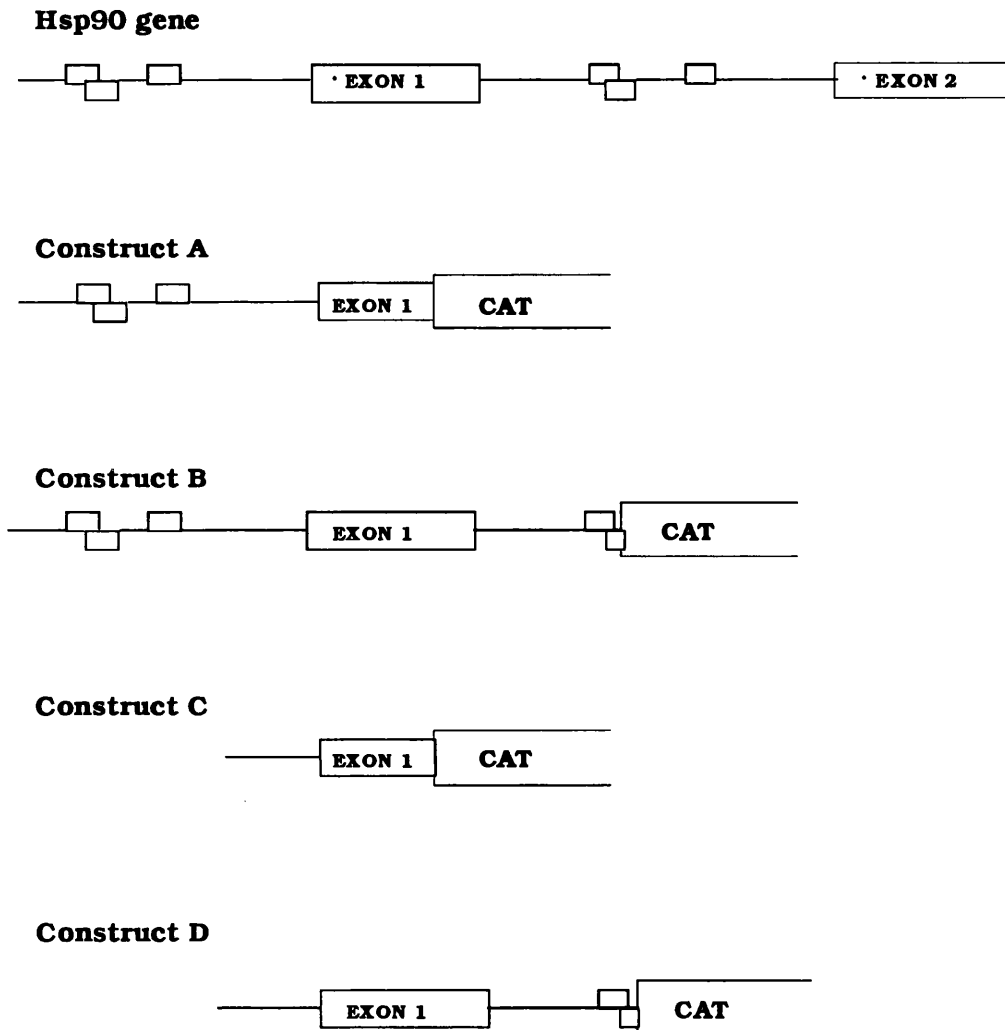
Construct D

Fig.2.2.2. A schematic representation of the four Hsp90 promoter constructs used in these experiments. The human Hsp90β gene has been included in the figure to allow comparison with the constructs. Construct B contains the full length promoter sequence, while A and D contain the upstream and intronic regions respectively. Construct C does not contain heat shock elements. (Heat shock elements are represented here by small boxes). The Hsp90 protein has been replaced by a reporter chloramphenicol acetyltransferase gene to facilitate investigation of promoter activity.

and does not contain any HSES. All four constructs are capable of driving the expression of the CAT gene. A fifth CAT construct, IE, was used as a control. The IE construct contains promoter elements from herpes simplex virus immediate early genes and is not induced by heat shock.

(ii) Cell culture

The activity of the Hsp90 promoter constructs was tested in relevant human cell lines. Three cell lines were examined - the histiocytic lymphoma cell line U-937 (Sundström and Nilsson, 1976), T cell leukaemia-derived Jurkat E6-1 cells (Weiss et al., 1984) and B lymphoblast-derived Daudi cells (Klein et al., 1968). Cells were grown in RPMI 1640 media [Life Technologies], supplemented with 10% Fetal Calf Serum [Life Technologies], and subcultured while still in log phase, as determined by growth curves. Frozen stocks were generated from the initial cell cultures, and cells were not used for more than 30 passages.

(iii) Transfection of promoter constructs
(a) U-937 cells

Cells were grown to near-confluence, centrifuged at 500g for 10 minutes at 4°C and resuspended in ice-cold PBS. Cell viability was tested by adding an equal volume of 0.2% w/v Trypan blue solution [Sigma] to 25µl resuspended cells and counting the proportion of live cells using a haemocytometer. The cells were respun and resuspended to a final concentration of 8×10^7 viable cells/ml in PBS and kept on ice until required. For each transfection 500µl of cell suspension was used in a 0.4cm electroporation cuvette [Bio-Rad]. Promoter construct DNA was purified as described above and sterilised by ethanol precipitation prior to transfection. Approximately 15µg DNA was added and after mixing the cells were electroporated at 960FD, 220V in a Bio-Rad Genepulser [Bio-Rad]. Immediately following electroporation 1ml of serum-supplemented media was added and the mixture transferred to a 50ml flask containing

10mlgrowth media, preheated to 37°C. The solution was agitated to remove clumps and cells were allowed to recover in an incubator for 48 hours. Cells were then transferred to 50ml Falcon tubes and centrifuged at 500g for 10 minutes at 4°C. The pellets were resuspended in 5ml ice-cold PBS, recentrifuged, harvested in 1ml PBS. and stored at -20°C until required.

(b) Jurkat cells

Cells were grown in continual log phase and kept as near as possible to 2×10^5 viable cells/ml on the day of transfection. The suspensions were centrifuged as for U-937s and the cells resuspended to a concentration of 8.75×10^7 cells/ml in Iscove's Medium [Imperial, Andover, Hampshire]. Eight hundred microlitres of the cell suspension was placed in a 0.4ml electroporation cuvette with 20µg sterile plasmid DNA and left to stand at room temperature for 5-10 minutes. The cells were electroporated at 960FD, 300V and left to stand for five minutes at room temperature. Cells were transferred to a flask containing 10ml preheated serum-supplemented growth media and incubated at 37°C for 48 hours before harvesting in PBS as for U-937s.

(c) Daudi cells

Cells were grown as for Jurkats, centrifuged at 500g for 10 minutes and resuspended to a final concentration of 3.2×10^7 viable cells/ml in RPMI 1640/10% Fetal Calf Serum supplemented with 25mM Hepes and 1mM penicillin/streptomycin [Life Technologies]. For each transfection 500µl of suspension was placed in a 0.4cm electroporation cuvette with 10µg of plasmid DNA and left on ice for 20 minutes. Cells were electroporated at 960FD, 250V and added to a flask containing 10ml conditioned media (5ml supernatant from the initial centrifugation of cells + 5ml fresh RPMI 1640/10% Fetal Calf Serum). Cell suspensions were incubated and harvested in PBS as above.

(iv) Heat shocking of transfected cells

Cells were allowed to recover for 24 hours in a 37°C incubator after electroporation. Heat shocking of cells was carried out by placing flasks in a 42.5°C preheated water bath for 40 minutes. Following heat shock cells were returned to the 37°C incubator and allowed to recover and grow for a further 48 hours.

(v) Plasmid uptake assay

Cells were centrifuged at 1500g, the PBS discarded and the pellets resuspended in 0.25M Tris-HCl (pH7.8). Cells were lysed by freeze-thawing three times in liquid N_2 and the cellular debris centrifuged down and discarded. The transfected cell lysate was incubated with 100mg/ml RNAase A at 37°C for 30 minutes, followed by incubation with 1mg/ml Proteinase K, also for 30 minutes at 37°C. Two volumes 20x SSC were added and the lysate transferred to a Hybond N nylon membrane using the Bio-Dot slot blotter. The DNA was cross-linked to the membrane in a UV Stratalinker and prehybridised in Southern prehybridisation mix (appendix 1) in a Hybaid MAXI oven [Hybaid] at 65°C overnight. A probe containing ^{32}P-labelled chloramphenicol acetyl transferase (CAT) DNA was made (see section (v) below) and hybridised to the membrane overnight at 65°C. The membrane was washed with 2X SSC, 0.1% SDS on a 42°C shaking platform, and exposed to XAR fast film overnight at -70°C. Plasmid uptake was equalised by scanning densitometry.

(vi) Preparation of CAT probes

Plasmid DNA was cut with appropriate restriction enzymes and buffers and run out for 1-2hrs on a 1% low melting point agarose [Life Technologies]/TAE (appendix 1) gel. The desired fragment was cut out from the gel and the plasmid DNA extracted using the Geneclean II kit [Stratech, Luton], following the recommended protocol. The DNA concentration was determined by

spectrophotometry, and the purified DNA stored at -20°C until required. A probe was made by adding 1-2μg plasmid DNA to a solution containing 10μl oligonucleotide labelling buffer (appendix 1), 2μl ^{32}P-dCTP and 0.5μl DNA polymerase I [Life Technologies]. The volume was adjusted to 50μl with dH_2O and the probe was passed through a 1ml column of Sephadex G-50 [Pharmacia] that had been equilibrated with 2 x SSC. The probe was boiled for 10 minutes and added to the filters containing the prehybridisation solution.

(vii) Chloramphenicol acetyltransferase (CAT) assay

CAT assays were conducted according to the procedure of Gorman (Gorman, 1985). Reaction mix consisting of 70μl 0.25M Tris-HCl (pH 7.8), 0-35μl dH_2O, 5-50μl transfected cell lysate, 20μl 4mM acetyl CoA [Boehringer] and 1μl D-Thero-(dichloroacetyl-1,2-^{14}C) chloramphenicol (1μCi/μl [Dupont]) was incubated for 30-60 minutes in a 37°C water bath. The amount of cell lysate added was determined by equalisation for results obtained by the plasmid uptake assay. The chloramphenicol was extracted with 1ml ethyl acetate [BDH] by vortexing each reaction for at least 30 seconds and centrifuging for 5 minutes at 500g. The upper organic phase was retained and evaporated under vacuum. The pellets were resuspended in 40μl ethyl acetate [BDH] and spotted onto silica gel thin layer chromatography plates [Whatman]. The plates were subjected to ascending chromatography in a tank containing a 95:5 mixture of chloroform/methanol, air-dried and exposed to XAR fast film for 1-3 days.

The visible spots were used to determine the position of the converted chloramphenicol in each sample, and the relevant spots were cut out from the silica gel, and placed in a scintillation vial. To these vials were added 5ml of EcoScint scintillant [BDH]. The vials were subsequently placed in an Intertechnique SL30 scintillation counter [LabLogic, Sheffield] and the radiation counts quantified. The ratio of converted to unconverted chloramphenicol was regarded as a measure of promoter construct activity within the cells.

CHAPTER 3: HSP EXPRESSION IN MRL/*LPR* MICE

3.1. Detection of hsps

For detection of heat shock proteins using acrylamide gels, specific antibodies were needed to recognise the appropriate protein. Antibodies used in these experiments recognised the relevant heat shock protein when the purified protein was run on an acrylamide gel and bound to labelled antibody using Western blotting and ECL (Fig.3.1.1).

Quantitation of hsps

Accurate assessment of heat shock protein levels in tissues requires an invariant factor that can be utilised to gauge inter-sample variation, and hence used to equalise for protein loading. The method routinely used in our laboratory was developed by Norton *et al* (Norton *et al.*, 1989). This method utilises levels of actin on a Coomassie Blue-stained gel as determined by measuring the intensity of the protein band by scanning densitometry. The density of the hsp band in the autoradiograph of each sample is adjusted for actin content to obtain an accurate estimate of the relative hsp value. Actin has been shown to be an accurate indicator of total cell protein, rising linearly in association with an increase in total cell protein levels (Dhillon, 1993). Detection of specific proteins is readily achieved using a monoclonal antibody to the desired protein and enhanced chemiluminescence (ECL, see Chapter 2). Linearity of ECL on autoradiographs was determined using liver samples from control mice (Fig.3.1.2). and subsequent detection of hsps was carried out within the linear range.

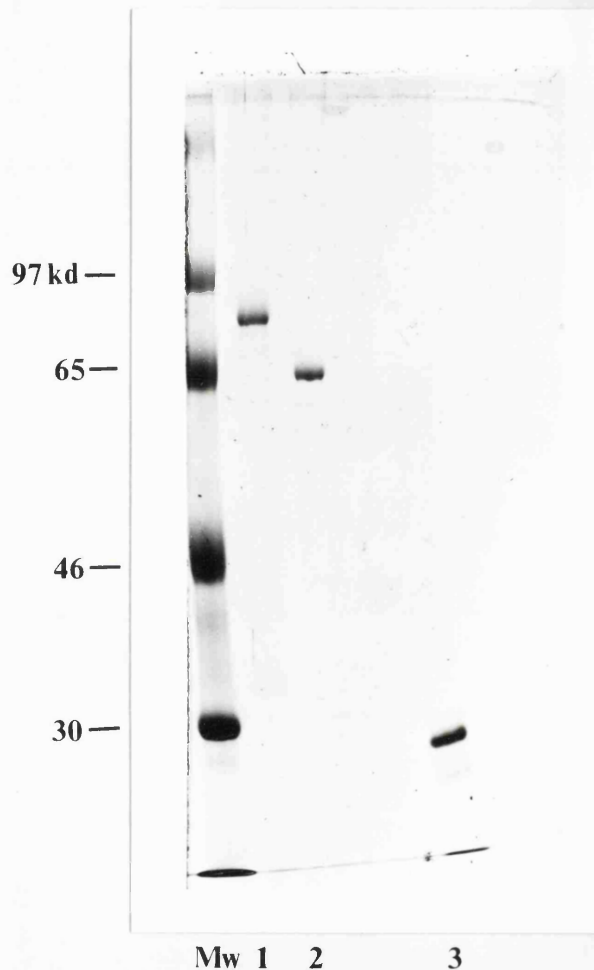## Specific antibodies recognise purified heat shock proteins using Western blotting techniques

Fig.3.1.1. The antibodies used recognise specific heat shock proteins using Western blotting techniques. Individual heat shock proteins (5μg) were run on separate tracks of an SDS-PAGE gel, blotted onto nitrocellulose, and probed with an appropriate antibody. Ac88 recognises purified Hsp90 (Lane 1) while SPA-801 recognises Hsp70 (Lane 2). The band visible in Lane 3 is another heat shock protein, Hsp27, detected using the monoclonal antibody D5 (a gift from Dr R. King). The molecular weights markers used are Rainbow markers, which remain visible after transfer to nitrocellulose.

Determination of the linear range for detection of proteins
using enhanced chemiluminescence
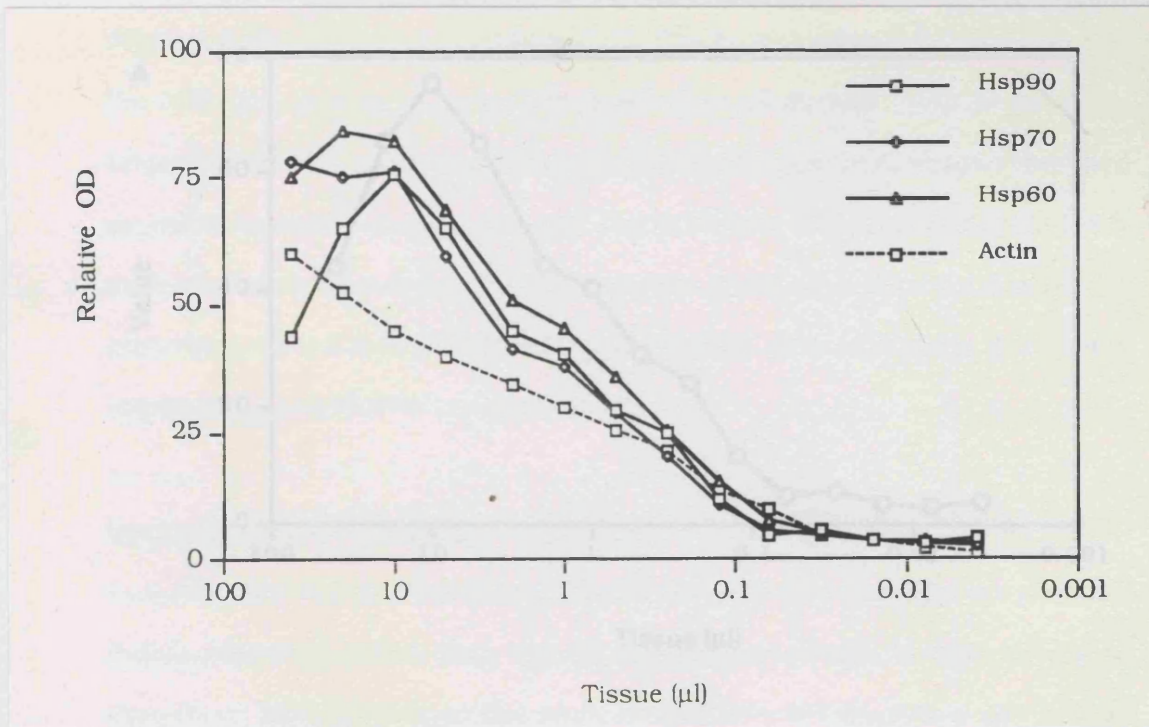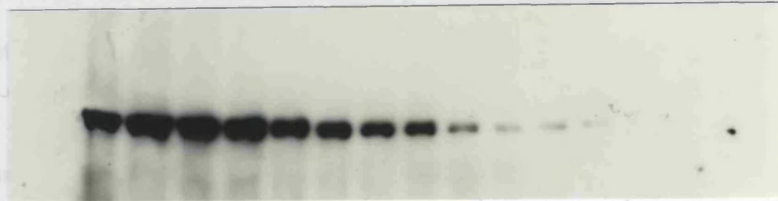
Fig.3.1.2. Determination of the linear range for ECL was carried out using serial dilutions of liver samples and relevant anti-hsp antibodies. Subsequent experiments were carried out ensuring that results were within this range. Values obtained from densitometric analysis of the Hsp90 bands shown in 3.1.2B have been plotted in 3.1.2A, with results from Hsp70 and Hsp60 blots also plotted in 3.1.2A. The linearity of actin bands on SDS-PAGE gels after Coomassie Blue staining is also shown in Fig 3.1.2A. after adjustment to the appropriate scale.

3.2. Expression of Hsp90 in MRL/lpr mice

In an initial study, spleens from age-matched MRL/lpr, MRL/++, and Balb/c mice were tested for Hsp90 protein levels. These mice were 6 months old, and the MRL/lpr mice were in an advanced stage of disease, with proteinuria, extensive hair loss, lymphoproliferation, and skin lesions. In contrast, there were no visible signs of disease in the MRL/++ or Balb/c mice. Hsp90 protein levels were clearly elevated 2-3 fold in the spleens of MRL/lpr mice compared with control mice (Fig.3.2.1). MRL/++ mice, in contrast, showed no such elevation of Hsp90 compared with Balb/c controls.

Expression of Hsp90 in spleen

To further investigate expression of Hsp90 nine MRL/lpr and eight age-matched Balb/c mice were culled, their spleens removed and protein samples generated from them. MRL/lpr mice in this study ranged from 5-7 months of age, and all showed symptoms of active disease. Overexpression of Hsp90 was observed in the autoimmune strain compared with Balb/c controls (Fig.3.2.2). The mean Hsp90 value, in arbitrary units, was 2.37 ± 1.04 for the MRL/lpr mice, compared with the mean value for Balb/c mice of 0.99 ± 0.95. Thus there appears to be a link between overexpression of Hsp90 and active disease in the autoimmune mouse strain.

Expression of Hsp90 in spleen during development

Overexpression of Hsp90 was observed in mice as young as 3 months of age (Fig.3.2.3A), persisting through the course of disease until death, which was usually from kidney failure. In contrast, levels of Hsp90 remained fairly constant throughout the course of development in Balb/c mice, remaining at a level comparable to the healthy 1 month old MRL/lpr mouse. In the first two weeks post-partum Hsp90 was expressed at a high level in both strains compared with

later in development; this may indicate a role for Hsp90 in the developing mouse (Fig.3.2.1).

Hsp90 expression in other tissues

The initial observation that Hsp90 protein levels were elevated in spleens of MRL/lpr mice raised the question of specificity of this overexpression. To further examine the role of Hsp90 in diseased MRL/lpr mice the study was broadened to include a range of tissues. Kidneys were an obvious choice for investigation, since MRL/lpr mice typically die from renal failure. As work on human patients has shown that Hsp90 overexpression is linked to cardiovascular and/or central nervous system disease (Dhillon et al., 1994) brain (see Fig.3.2.3B) and heart were also selected for study. Liver, pancreas, gastric antrum, salivary glands and lung were used as controls and to complete the general survey of tissues. The thymus is severely atrophied in MRL/lpr mice (Andrews et al., 1978), and insufficient tissue was able to be obtained from individual mice for examination using the Western blotting protocol described in this thesis. No additional tissues were shown to have significantly increased Hsp90 levels, although the salivary glands had greater levels of Hsp90 in MRL/lpr mice than in control mice. This increase failed to reach statistical significance, however, and the spleen remained the only tissue with significantly elevated Hsp90 levels (Fig.3.2.4).

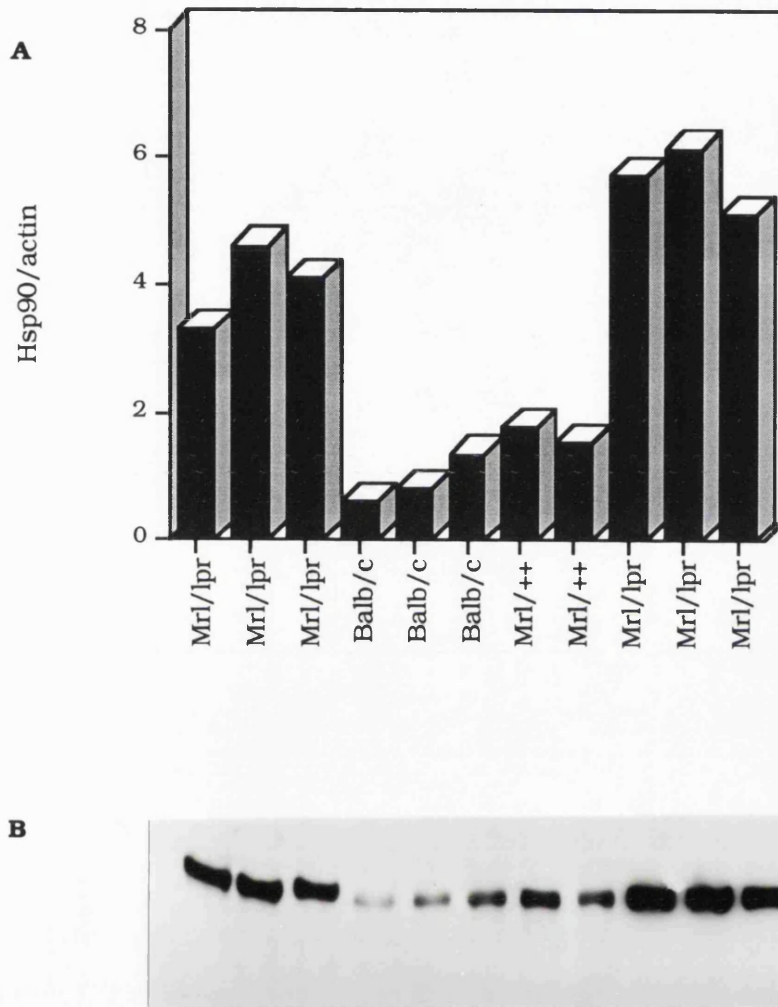## Hsp90 protein levels are elevated in MRL/*lpr* mice

Fig.3.2.1. An initial experiment revealed overexpression of Hsp90 in MRL/*lpr*
mice compared with Balb/c and MRL/++ controls as demonstrated by Western
blotting. The graph and corresponding photograph show individual age-matched
mice.The values for the Hsp90 band (H) from one half of an SDS-PAGE gel were
divided by the values obtained from the actin band (A) from the destained
Coomassie Blue-stained gel. i.e. H/A. These values were equalised for inter-gel
differences using the H/A value from a cell line standard run with each gel.
Elevated Hsp90 levels are clearly visible in the MRL/*lpr* mice with active disease
compared with healthy controls. Hsp90 values on the y-axis are arbitrary units.
The bands on the left-hand side of the photograph are from 6-month old mice;
the three MRL/*lpr* bands on the right hand side of the photograph are from 2-3
week old mice, indicating high levels of Hsp90 at an early age. However,
insufficient tissue was able to be obtained from young Balb/c mice for
comparison.

Hsp90 is elevated in spleens of MRL/*lpr* mice compared with age-matched Balb/c controls.
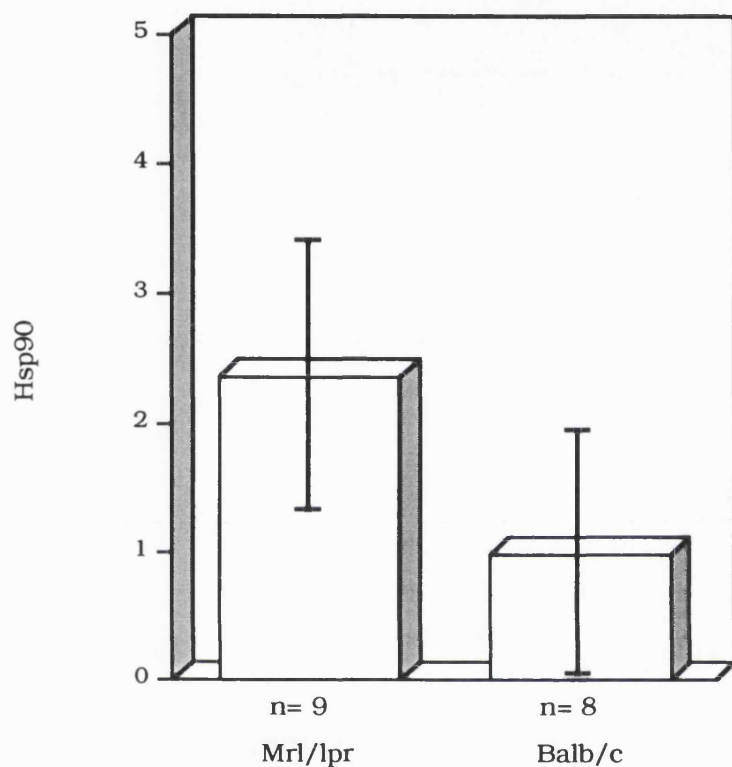
Fig.3.2.2. In a survey of spleens of MRL/*lpr* mice with active SLE-like disease Hsp90 protein is significantly overexpressed compared with levels of protein expression observed in healthy age and sex-matched Balb/c controls (p<0.05). The mice used in this survey were 6-month-old females, and all MRL/*lpr* mice showed signs of active disease, including proteinuria, skin lesions and extensive hair loss. The graph depicts the mean Hsp90 value obtained from the MRL/*lpr* mice examined expressed as a percentage of the mean value obtained from the Balb/c mice.

Hsp90 expression is elevated from an early age in spleens of MRL/*lpr* mice, but not in other tissues
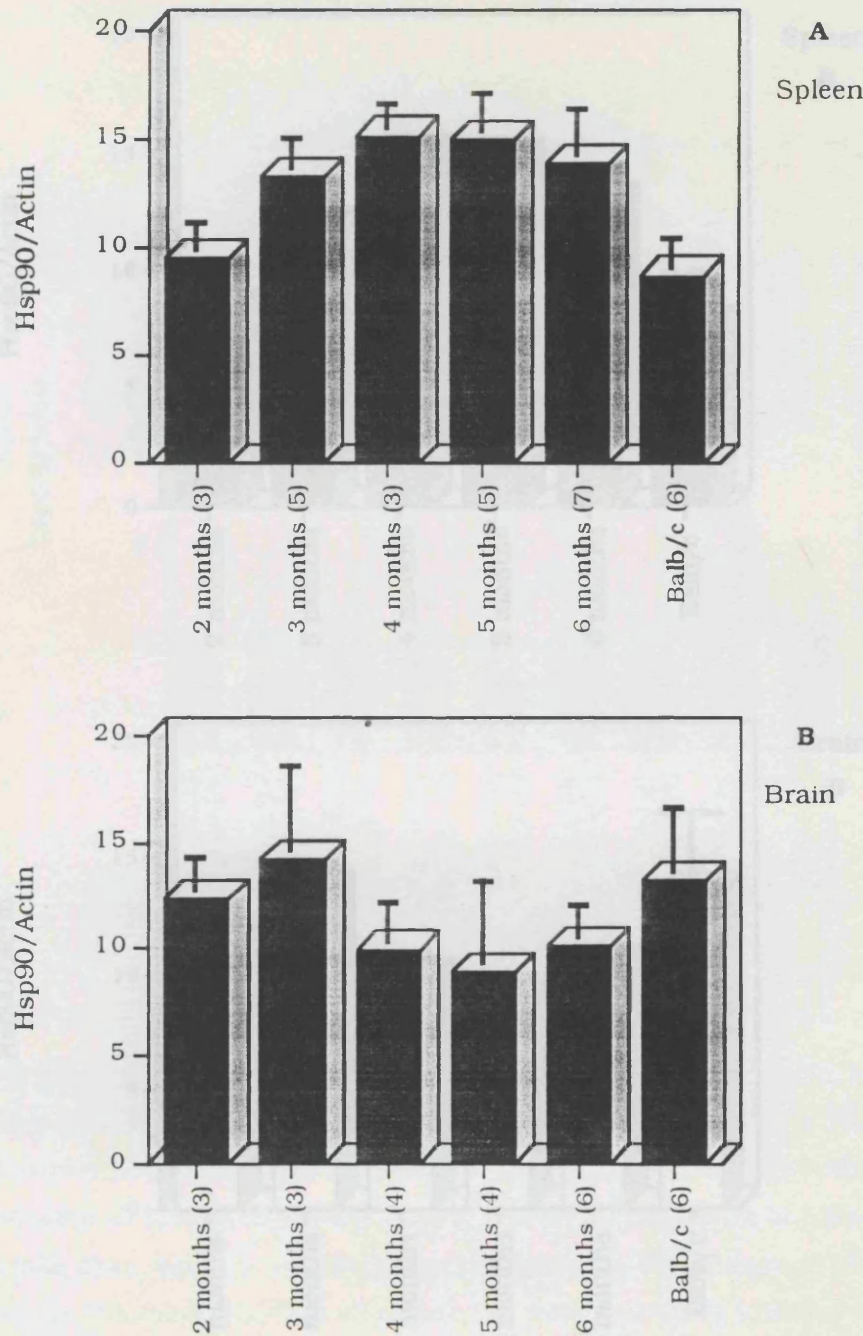
Fig.3.2.3. (A) Hsp90 levels rise with age and hence progression of disease in spleens of MRL/*lpr* mice, with significantly elevated levels of protein observed from three months (p = 0.046). However, this rise is not mirrored in other tissues. Brain (B) is a typical example of expression patterns obtained from other tissues. The graph depicts the mean Hsp90 value from the mice examined (numbers are given in parentheses) with standard deviations shown as error bars.

Hsp90 levels in tissues of MRL/*lpr* mice compared with Balb/c controls

Hap90/Actin

Kidney (p=.0986)
Brain (p=.1620)
Heart (p=.7176)
Pancreas (p=.2042)
Gastric Antrum (p=.2932)
Salivary (p=.2932)
Lung (p=.4413)
Spleen (p=.0421)
Liver (p=.2417)

Balb/c (6) Mrl/lpr (6)
Balb/c (5) Mrl/lpr (6)
Balb/c (6) Mrl/lpr (6)
Balb/c (4) Mrl/lpr (5)
Balb/c (5) Mrl/lpr (5)
Balb/c (6) Mrl/lpr (3)
Balb/c (6) Mrl/lpr (4)
Balb/c (8) Mrl/lpr (9)
Balb/c (7) Mrl/lpr (8)

Fig.3.2.4. Studies on a range of tissues in MRL/*lpr* and Balb/c mice reveal elevation of Hsp90 in the autoimmune strain to be a tissue-specific phenomenon, localised to the spleen. This observation supports the notion that elevation of this protein may have a role in pathogenesis of MRL/*lpr* disease, rather than being a simple stress response to the disease state. The graph depicts the mean Hsp90 value from the mice examined (numbers are given in parentheses) with standard deviations shown as error bars. Results from Mrl/*lpr* mice are expressed as a percentage value, with the Hsp90/Actin value from the control Balb/c mice defined as one unit.

3.3. Expression of Hsp70 in MRL/*lpr* mice

The availability of distinct antibodies facilitated examination of the expression patterns of the constitutive Hsp73 and the inducible Hsp72 proteins in MRL/*lpr* mice. A small proportion of SLE patients (approximately 10%) are known to overexpress Hsp72, but no such overexpression has been observed with Hsp73 (Dhillon *et al.*, 1991). It has also been reported that Hsp70 is overexpressed in kidneys of MRL/*lpr* mice in an age-dependent manner (Deguchi. 1991). To further investigate similarities between murine and human lupus tissue samples from MRL/*lpr* and Balb/c mice were probed for the two Hsp70 proteins.

<u>Hsp72</u>

Hsp72 was not detectable in all tissues studied by the methods used in this thesis. However, four tissues - liver, kidney spleen and brain - had protein levels sufficiently high for observation. Fig.3.3.1. shows the typical pattern of Hsp72 expression seen in liver, with little protein observed in early stages of development but an increasing amount of Hsp72 becoming noticeable as the disease progresses. Kidney and spleen had similarly increasing Hsp72 protein levels from 4-6 months of age. No clear elevation was seen in brain tissue, although there appeared may have been slight upregulation of the protein (see Fig.3.3.2). However, in this tissue the highest levels of Hsp72 were detected in the 2-month MRL/*lpr* mouse rather than the diseased 6-month animal. Hsp72 overexpression could thus be a result of the stress of chronic disease, while Hsp90 overexpression, which appears to be limited to the spleen, might be a disease-specific phenomenon that in turn could have some role in the disease process. Overexpression of Hsp72 appears to lag behind other features associated with disease, e.g. production of anti-DNA antibodies, proteinuria and lymphoproliferation.

Hsp73

Under conditions of stress the constitutive member of the Hsp70 family, Hsp73, is only slightly upregulated. However, the protein is expressed at high levels in normal tissue. In the MRL/*lpr* mice levels of Hsp73 showed little variance from Balb/c controls (Figs 3.3.3. and 3.3.4), and no tissue in the autoimmune mouse had significantly elevated protein levels. The levels of Hsp73 in heart tissue were somewhat higher in the MRL/*lpr* mice compared with Balb/c mice, although this elevation failed to achieve statistical significance.

3.4. Expression of Hsp60 in MRL/*lpr* mice

As with the levels of Hsp73, little variation in the expression of Hsp60 was observed in MRL/*lpr* mice compared with Balb/c controls. There was no overexpression in any of the tissues investigated, and protein levels remained relatively constant as the mice aged. Fig.3.4.1. shows a representative result obtained from MRL/*lpr* and Balb/c liver samples. Levels of Hsp60 expression are comparable in young and old MRL/*lpr* mice and in control Balb/c mice. Similar patterns were observed in the other tissues investigated. These results have been compiled in Fig.3.4.2. While a functional role for Hsp60 cannot be ruled out in the development of MRL/*lpr* disease on the basis of this experiment, any such role does not result in increased expression of the Hsp60 protein. This experiment does not preclude altered compartmentalisation of the protein, for example, or expression on the cell surface.

Hsp72 protein levels increase with age in the livers of MRL/*lpr* mice compared with Balb/c controls
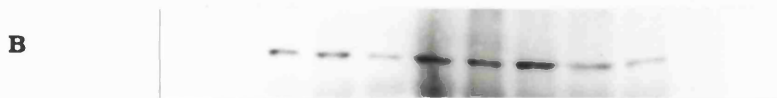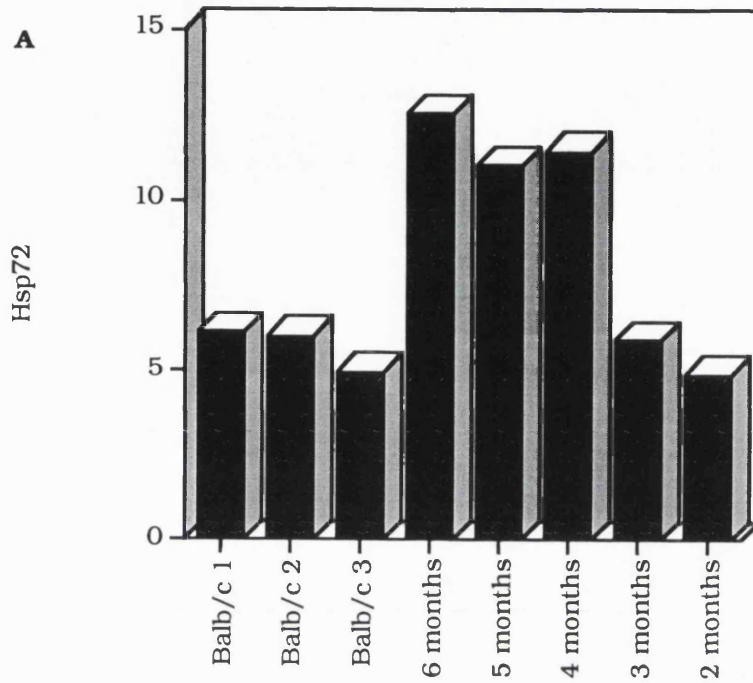
Fig.3.3.1. Hsp72 protein levels appear to increase in the livers of MRL/*lpr* mice with the onset of disease, and are elevated from four months (p = 0.043, n= 5 mice) compared with healthy Balb/c mice (n= 5). The graph (A) and corresponding photograph (B) are results obtained with individual mice (N.B. The ages of the MRL/*lpr* mice, labelled as months, *decrease* from left to right),
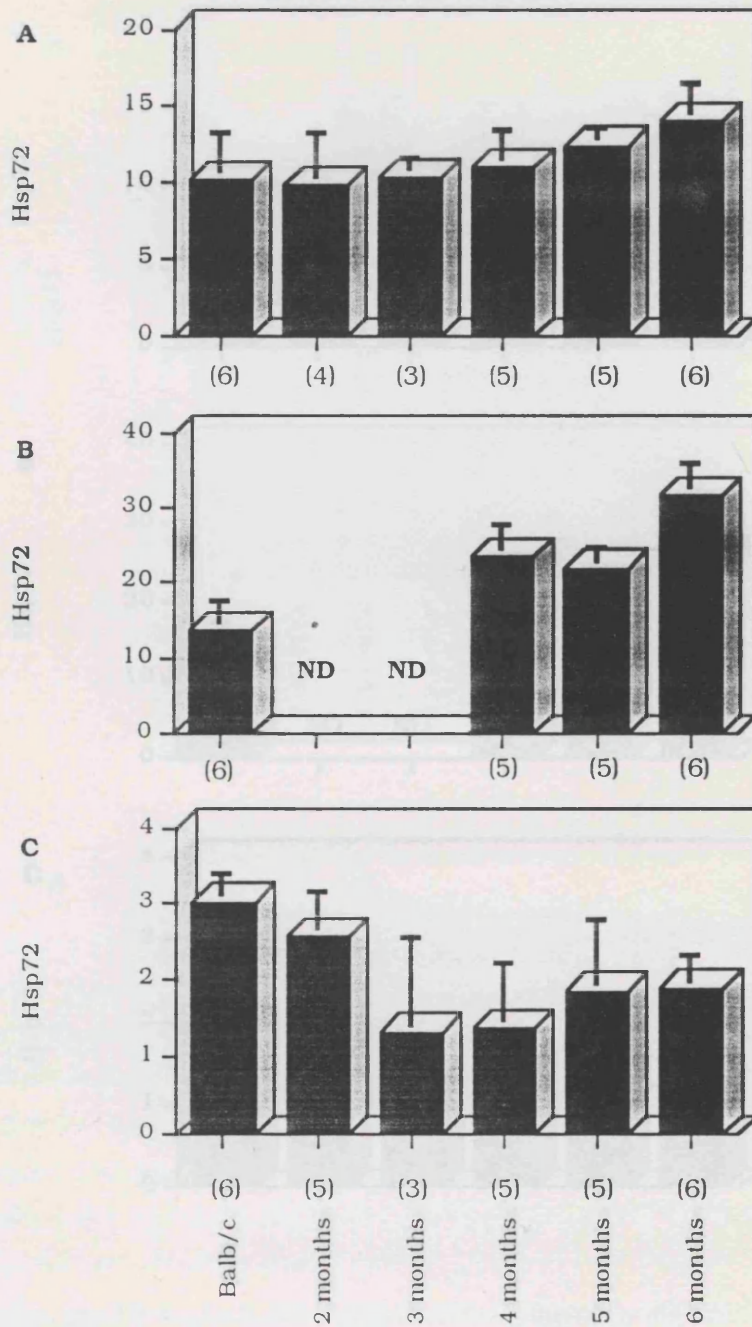
Fig.3.3.2. Increased stress may result in increased systemic levels of Hsp72. In the spleen (3.3.2B) the protein levels increased with onset of disease ($p = 0.037$) although the increased levels failed to reach statistical significance in the kidney (3.3.2A, $p = 0.062$). The levels of Hsp72 in the brains (3.3.2C) of MRL/*lpr* mice were lower than that of healthy controls. (ND = Not determined).

Hsp73 shows no overexpression in the kidneys of MRL/*lpr* mice compared with Balb/c controls
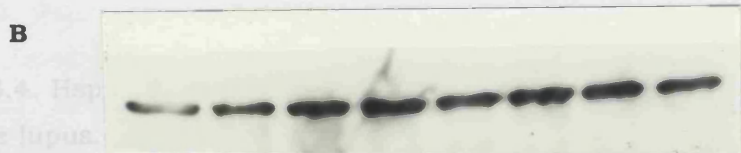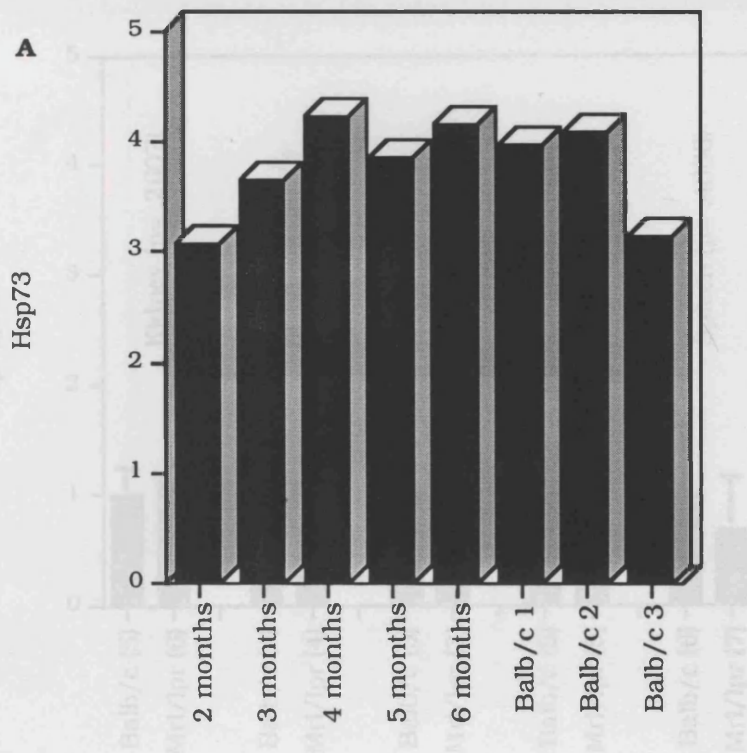
Fig.3.3.3. Hsp73 levels do not appear to be significantly elevated in the tissues of MRL/*lpr* mice compared with healthy controls, as illustrated by the kidneys of individual mice in this figure. There may be a slight increase in the levels of this protein with increasing age, although levels remain comparable to the protein levels of healthy mice.
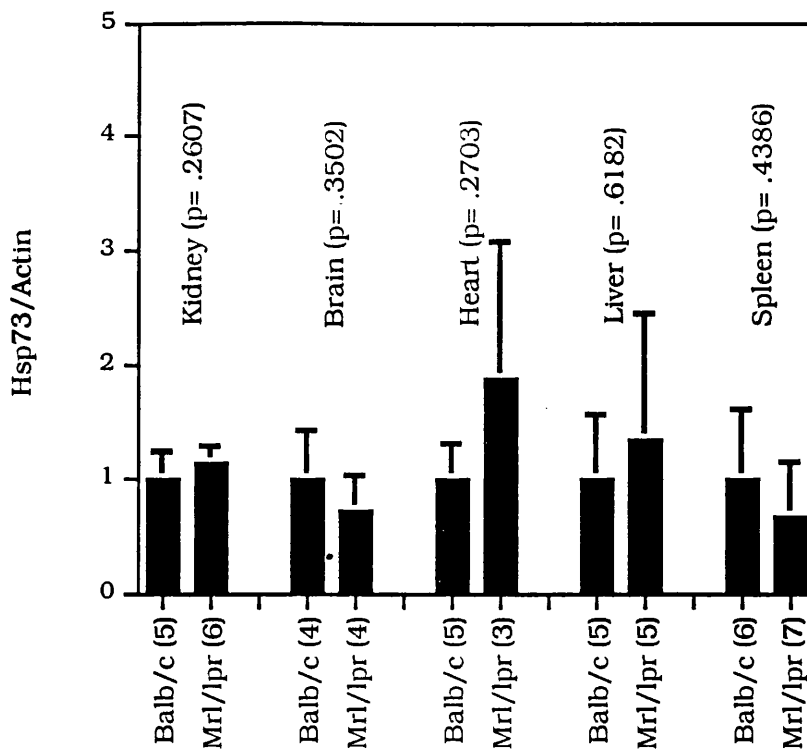
Fig.3.3.4. Hsp73 protein levels do not appear to be significantly affected by murine lupus, and appear to be similar in 6-month-old age-matched MRL/*lpr* and Balb/c mice. Heart tissue may be an exception to this, although the greater levels detected in MRL/*lpr* mice compared with healthy controls failed to reach statistical significance. The graph depicts the mean Hsp73 value from the MRL/*lpr* mice examined expressed as a percentage of the mean Hsp73 value obtained from control Balb/c mice (numbers are given in parentheses). Standard deviations are shown as error bars.

No overexpression of Hsp60 is observed in the liver of MRL/*lpr* mice compared with Balb/c controls

A

B

Fig.3.4.1. There appears to be little change in levels of Hsp60 detected in the livers of MRL/*lpr* mice with increasing age and hence onset of disease, these levels remaining fairly constant and comparable to the levels detected in healthy Balb/c mice. Results obtained from individual mice are depicted in this illustration.

Hsp60 is not overexpressed in MRL/*lpr* mice
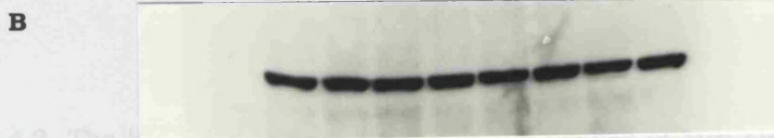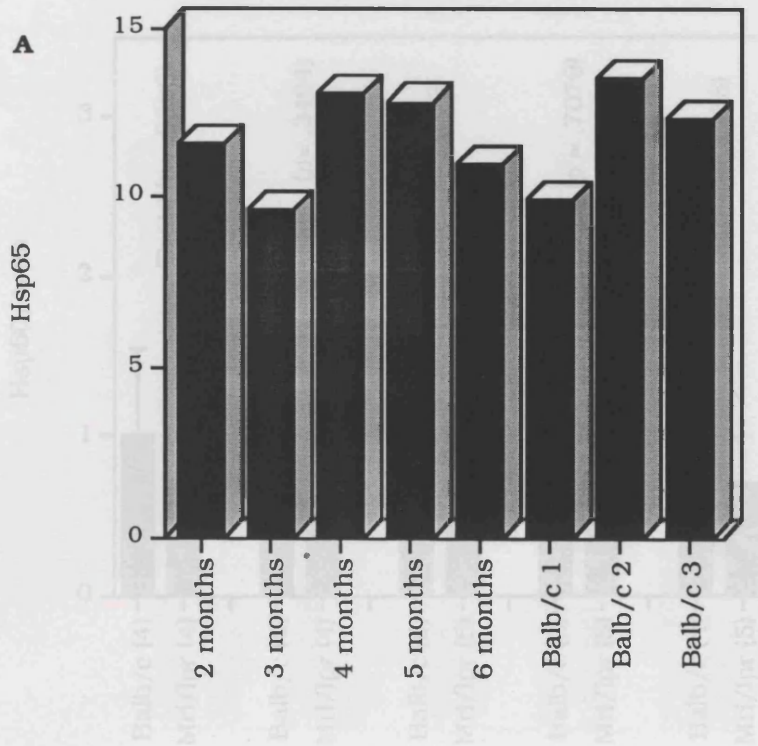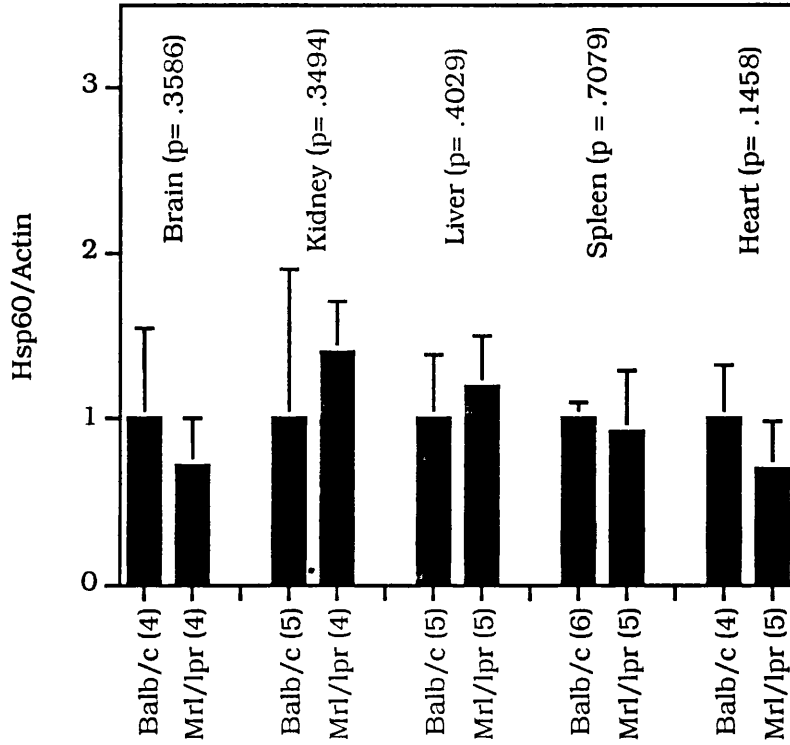
Fig.3.4.2. The levels of Hsp60 detected in the tissues of MRL/*lpr* mice do not appear to be significantly affected by disease, and are not significantly different from levels detected in healthy age-matched Balb/c mice. The graph depicts the mean Hsp60 value from the MRL/*lpr* mice examined expressed as a percentage of the mean Hsp60 value obtained from control Balb/c mice (numbers are given in parentheses). Standard deviations are shown as error bars.

3.5. Surface expression of Hsp90

Flow cytometry was conducted using a three-colour system to examine cell surface staining of Hsp90 in the spleens of five MRL/*lpr* mice. Phycoerythrin was conjugated with an antibody to a pan-B cell marker to distinguish these cells from T cells, another major sub-population of cells in the spleen. Fluorescein isothiocyanate was conjugated with Ac88 to investigate surface staining of Hsp90, while propidium iodide (PI) was used to mark dead cells. The proportion of T cells in the spleen is clearly evident in Fig.3.5.1., with a markedly reduced proportion of splenocytes staining positive for the B cell marker. A small number of cells in the MRL/*lpr* samples, is apparently staining positive for Hsp90 surface expression.

However, on addition of PI the subset of splenocytes staining positive for Hsp90 was shown to consist almost entirely of dead cells (Fig.3.5.2). Less than 0.25% of the live cells in MRL/*lpr* mice had significant surface expression of the heat shock protein, no greater than the proportion of cells staining positive for Hsp90 in either MRL/++ or Balb/c mice.

3.6. Transcription of hsps

The nuclear run-on assay is an excellent tool for investigating the rate of gene transcription. The technique involves blockage of transcription, removal of the pool of cytoplasmic unlabelled ribonucleotide and isolation of the nuclei. This has the effect of removing the large amount of completely transcribed ribonucleotide, and by restarting transcription from the isolated nuclei allows a thorough examination, by incorporation of radiolabelled nucleotides into the nascent RNA, of any active genes. The nuclear run-on technique has the added advantage that by using appropriate probes a number of genes can be examined simultaneously.

There are several possible mechanisms accounting for the overexpression of Hsp90. The protein may have an altered half-life so it remains for longer in the cell, hence increasing the total amount of this protein at any given moment without affecting transcription rates. Alternatively, the messenger RNA itself may not be degrading as rapidly as it would under normal conditions. In addition there may be an influx into the tissue of an abnormal subset of cells with high constitutive expression of the protein. A nuclear run-on assay allows the investigation of another possibility, i.e. that the rate of gene activity has been altered. In this manner, the enhanced expression of the Hsp90 gene would subsequently lead to overexpression at the protein level.

Several genes, including those of the major heat shock protein families, were examined for transcriptional activity in isolated nuclei. There was a slight increase in the levels of transcriptional activity measured from the plasmids containing the fragments of the Hsp90 genes, but no clear upregulation was noted. One of the fragments of the SmB gene was also slightly elevated in MRL/*lpr* mice compared with Balb/c controls, but another fragment from the same gene did not show the same increase. There were little differences in the transcriptional activity of the Hsp70, Hsp60 and Hsp27 genes of MRL/*lpr* and Balb/c mice.

Different proportions of B and T cells are evident in MRL/*lpr* mice
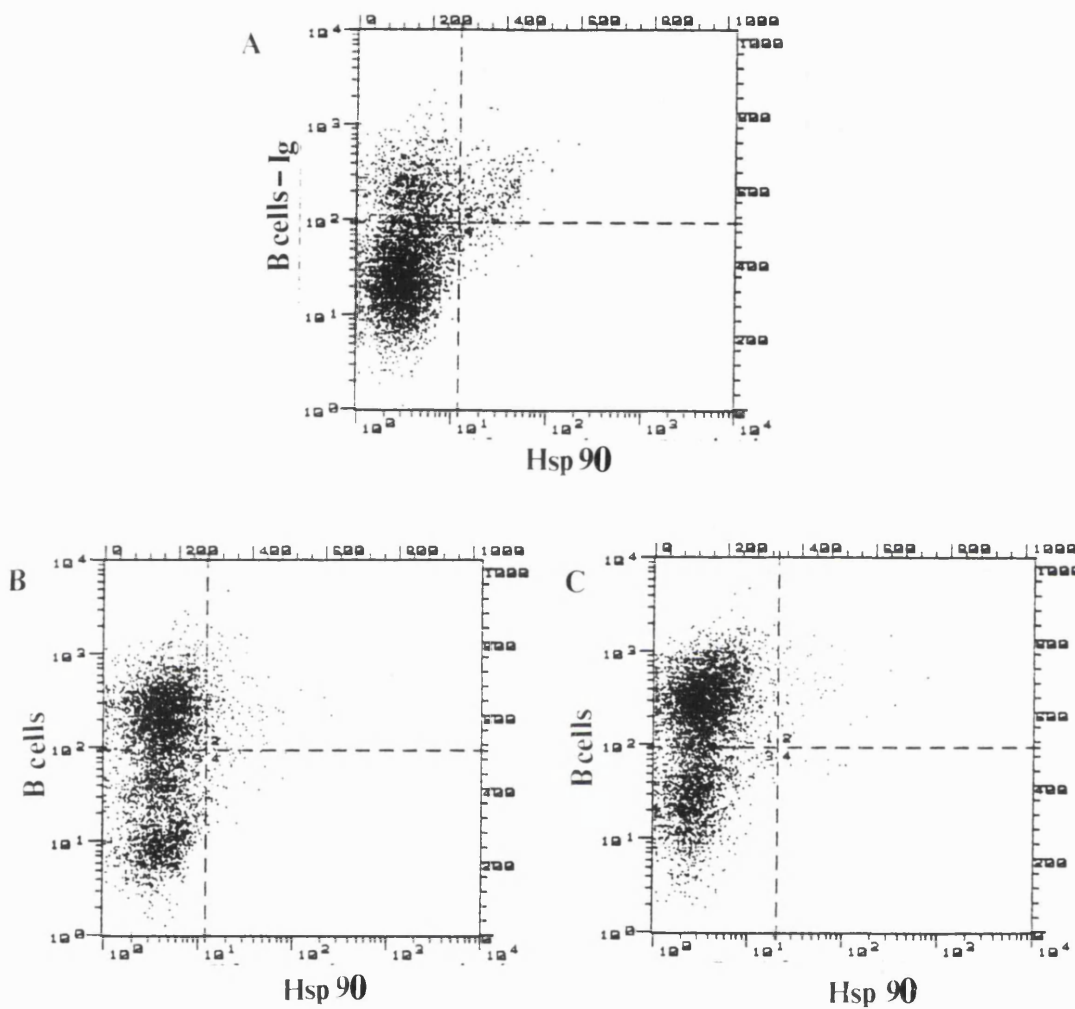
Fig.3.5.1. (A) A typical picture obtained from MRL/*lpr* mice shows some surface staining of Hsp90 in the splenocytes of these mice compared with Balb/c (B) and MRL/++ (C) mice. Note also the lower proportion of MRL/*lpr* cells staining positive for immunoglobulin, reflecting increased numbers of T cells in the MRL/*lpr* strain.
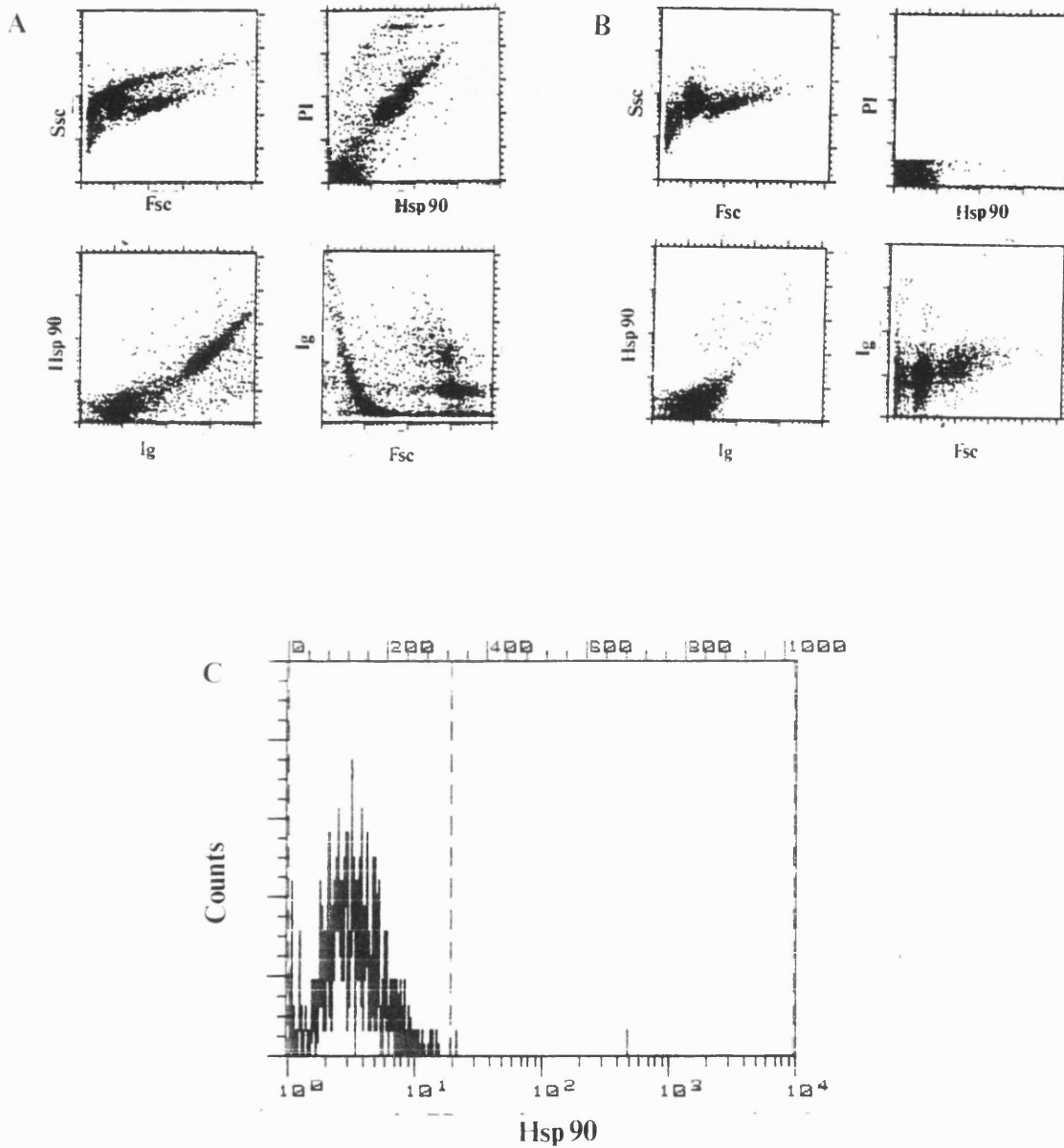
Fig.3.5.2. Expression of Hsp90 in splenocytes of MRL/*lpr* mice was revealed to be associated almost exclusively with dead cells. Cells staining positive for PI (A) were eliminated from the study (B), leaving less than 0.25% of the cell population staining positively for Hsp90 (C).

Transcriptional levels of Hsp90 and other hsps are unchanged in MRL/*lpr* mice
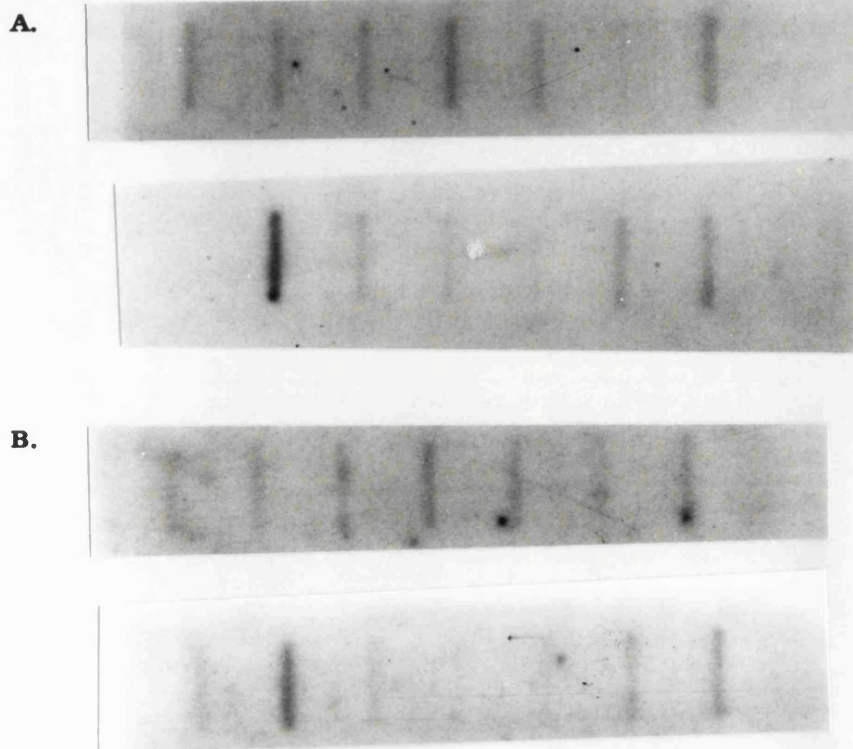
Table 3.6.

Plasmid	From	MRL/*lpr*	Balb/c	Plasmid	From	MRL/*lpr*	Balb/c
PJ3	Hsp90	13.74	10.89	2-3	Hsp70	10.49	11.38
90.5	Hsp90	12.45	10.58	p17	Hsp70	12.23	12.7
Hsp α	Hsp90	12.54	10.66	hsc70	Hsp70	10.36	11.30
90.3	Hsp90	12.71	11	DP8	Hsp70	11	11.15
SmB	SmB	12.41	10.87	pH8	Hsp27	11.77	11.51
PPI	SmN	11.67	11.15	pλ22A	Hsp60	11.34	11.63
201	SmB	11.46	11.48	123	Ribo	12.22	12.37

Fig.3.6.1. There appears to be little difference in the levels of heat shock protein transcription in MRL/*lpr* (Fig.3.6.3A) and Balb/c mice (Fig.3.6.3B). Table 3.6. shows the figures obtained from these experiments. The photographs read left to right across rows, corresponding to columns in the table. (Ribo = Ribosomes)

116

CHAPTER 4: DETECTION OF ANTI-HSP ANTIBODIES

4.1. Detection of antibodies by ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) is a versatile technique that is used for a wide range of purposes in immunology, including the detection of specific antibodies in an uncharacterised sample. The method is based on a colourimetric assay, using antibodies linked to an appropriate enzyme. The enzyme-linked antibodies will in turn catalyse a reaction to generate colour, which can be measured spectrophotometrically. An ELISA is relatively quick, simple and efficient, and has become one of the standard tools of immunological research.

For this study detection of anti-hsp antibodies in unknown sera was undertaken by coating flat-welled microtitre plates with the relevant heat shock protein and adding dilute sera to the coated plates. Any specific anti-hsp antibodies should thus have bound to coated hsp with great enough affinity to remain bound to the protein as non-specific antibodies were washed off during subsequent steps of the experiment. A blocking step was inserted prior to addition of sera, and this should have had the effect of eliminating or minimising non-specific binding to the plate. Furthermore, diluted sera was mixed with sera from another species to minimise many of the non-specific interactions which can cause high background readings. Sera from MRL/*lpr*, MRL/++, and Balb/c mice over a range of ages were used in these experiments (Table 4.1). While the MRL/*lpr* and Balb/c mice were age-matched the MRL/++ mice were considerably older, reflecting the desire to investigate the relevance of anti-hsp antibody levels to disease onset.

Mouse strain	No. tested	Age range (weeks)	Mean age (weeks)
MRL/*lpr*	41	3-38	19.5
MRL/++	17	3-70	39.8
Balb/c	46	3-39	18.33

Table 4.1. Mice used in ELISA experiments

Determination of optimal conditions for the assay was largely a matter of trial and error, and for the sake of brevity only a short discussion of the condition-setting experiments will be included in this thesis.

4.2. Detection of anti-DNA antibodies and total IgG assessment

Anti-DNA antibodies

Appraisal of disease severity in human SLE patients relies to a large extent on input from patients themselves, and no single clinical parameter can be said to accurately define the disease state. In a disease with similarly heterogeneous symptoms such as that of MRL/*lpr* mice, the problem of accurate assessment of disease severity is magnified, with no feedback possible from the mice! Thus a quantifiable clinical feature has to be chosen to give some indication of the condition of the mouse when samples are taken. The parameters selected for this project were anti-ssDNA and anti-dsDNA antibodies.

Generation of anti-dsDNA and anti-ssDNA antibodies in MRL/*lpr* mice are characteristic features of the murine disease, and significantly elevated titres of anti-ssDNA antibodies are seen from 2 months (Andrews *et al.*, 1978). Furthermore, presence of these autoantibodies correlates well with glomerulonephritis and mortality. Serum autoantibodies to DNA in young MRL/*lpr* mice at the pre-clinical or clinical stage are known to be predominantly

IgG (Slack *et al.*, 1984), and it was this class of antibody which was investigated most thoroughly in this project. Production of anti-dsDNA antibodies is somewhat retarded in comparison, although significantly elevated titres of these antibodies are found in all MRL/*lpr* mice by 4-5 months.

To enable direct comparison between anti-DNA antibodies and anti-Hsp antibodies the sera were initially tested for anti-ssDNA and anti-dsDNA antibodies. Mice maintained in our laboratory showed typical anti-ssDNA (Fig. 4.2.1) and anti-dsDNA (Fig. 4.2.2) profiles. The mean value obtained (in arbitrary units, based on the optical densities of the colour-developed ELISA) for anti-ssDNA antibodies from 19 MRL/*lpr* mice was 1.324, compared with 0.060 for age-matched Balb/c mice (n=35) and 0.189 for MRL/++ mice (n=17). Likewise the mean anti-dsDNA value from the same samples of MRL/*lpr* mice was 0.854, compared with 0.040 in MRL/++ mice. Anti-dsDNA antibodies were unable to be detected in 35 Balb/c mice using our ELISA system. These results are in agreement with established serological profiles of the respective mice strains (Theofilopoulos and Dixon, 1985).

Anti-DNA antibodies fall into four broad classifications:

(1)   Antibodies to dsDNA that are not cross-reactive with anti-ssDNA.

(2)   Antibodies reactive only to ssDNA, directed against DNA bases that lie hidden within the double helix of native, dsDNA.

(3)   Antibodies that react to both dsDNA and ssDNA.

(4)   Antibodies to Z-DNA. Z-DNA is formed by DNA helices coiling in an alternative, left-handed orientation opposite to that of normal DNA, which forms right-handed turns. The formation of Z-DNA may be associated with transcriptional activity (reviewed in Latchman, 1995).

Type (1) antibodies appear to be rare, and typical anti-dsDNA antibodies produced by MRL/*lpr* mice appear to be type (3), which presumably recognise the sugar-phosphate backbone of DNA. For this reason the high degree of correlation observed between anti-ssDNA and anti-dsDNA antibodies (Fig. 4.2.3)

is not unusual. The appearance of anti-dsDNA antibodies at different ages may reflect a change to the disease state, since anti-ssDNA antibodies are found in immunologically normal mice and pre-clinical MRL/*lpr* mice, whereas anti-dsDNA antibodies are relatively specific for disease. Specific anti-ssDNA antibody levels, i.e. those that could not be accounted for by anti-dsDNA titres, were not determined, although it is known that the number of anti-ssDNA secreting cells in spleens of MRL/*lpr* mice increases with age (Theofilopoulos and Dixon, 1985).

Total IgG levels

Total IgG levels were also measured to confirm that the MRL/*lpr* colony was typical. Increased immunoglobulin titres were evident from approximately eight weeks (Fig. 4.2.4.). The total IgG in older mice, ranging from 4-35 mg/ml, is in line with figures obtained in other colonies (Theofilopoulos and Dixon, 1985). Total IgG levels do not necessarily reflect the level of polyclonal activation, as there is a high incidence of monoclonal γ-globulins generated by these mice in the course of disease (Andrews *et al.*, 1978). However, IgG anti-ssDNA titres correlate well with polyclonal IgG activation (Izui *et al.*, 1978), so this parameter was subsequently used to check anti-hsp activity with polyclonal IgG activation.

Anti-ssDNA antibodies are found at high levels in MRL/*lpr* mice
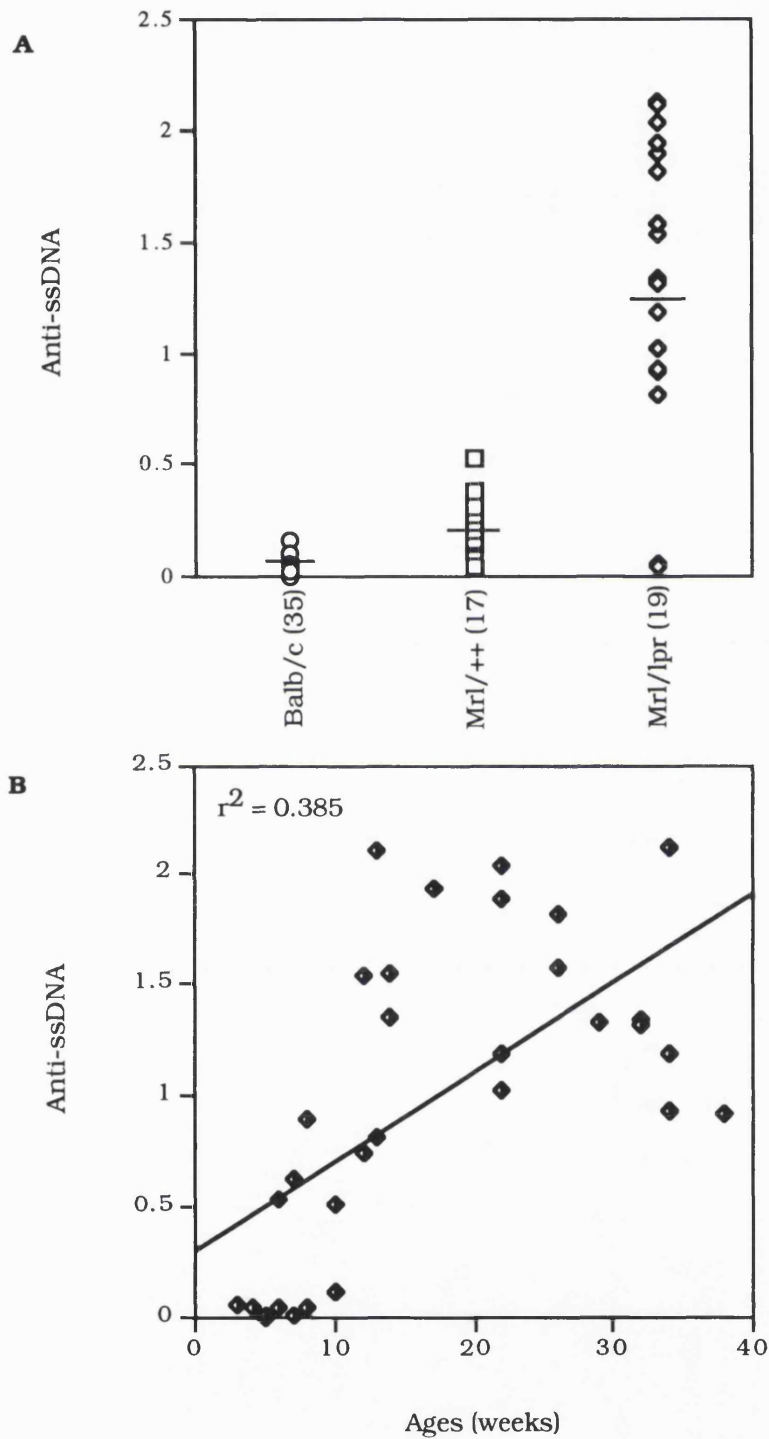
Fig.4.2.1. (A) MRL/*lpr* mice have significantly elevated levels of anti-ssDNA antibodies compared with MRL/++ (p= 0.003) and Balb/c (p< 0.001) control mice. (B) In MRL/*lpr* mice generation of these antibodies correlates well with increasing age and hence progression of disease.

Anti-dsDNA antibodies are generated later in the course of MRL/*lpr* disease than anti-ssDNA antibodies
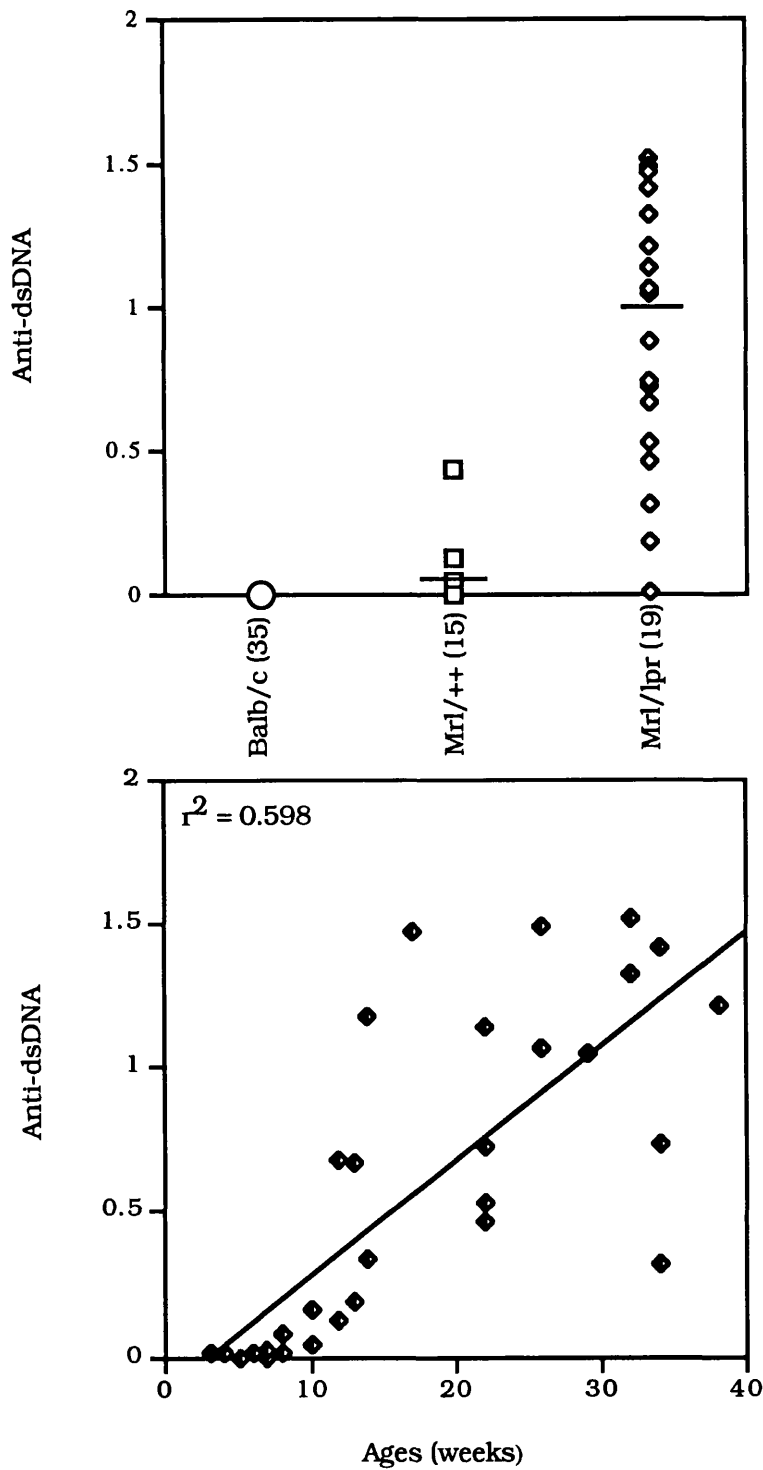
Fig.4.2.2. (A) Anti-dsDNA antibodies are a defining feature of Mrl/*lpr* disease, andlevels of these antibodies are significantly elevated compared with MRL/++ (p = 0.001) and Balb/c (p < 0.001) mice. (B). Production of these antibodies in MRL/*lpr* mice is retarded in comparison with anti-ssDNA antibodies, and titres are not significantly elevated until 3 months.

Production of anti-dsDNA and anti-ssDNA antibodies in
MRL/lpr mice shows significant correlation
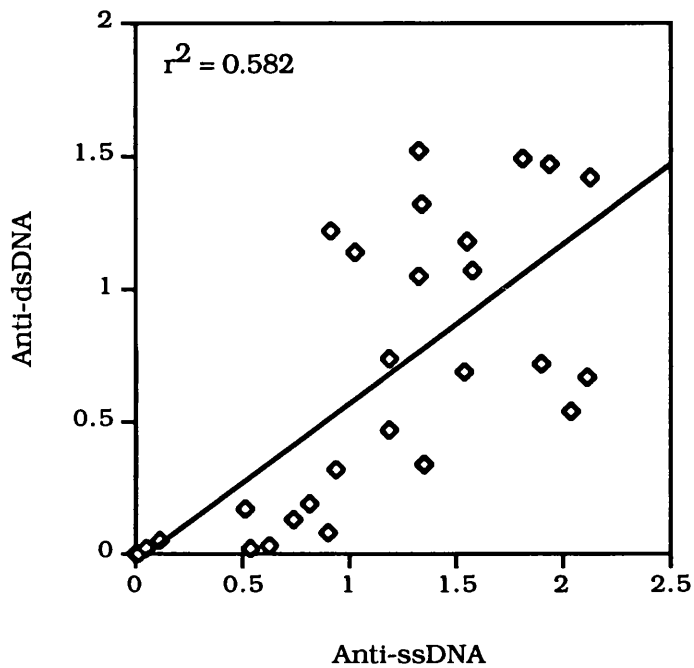
Fig.4.2.3. Anti-ssDNA and anti-dsDNA antibody levels in MRL/lpr mice show significant correlation (p = 0.009). This may be due to production of cross-reactive antibodies that recognise the sugar-phosphate backbone of DNA (see text).

Total immunoglobulin IgG levels rise with age in MRL/*lpr* mice
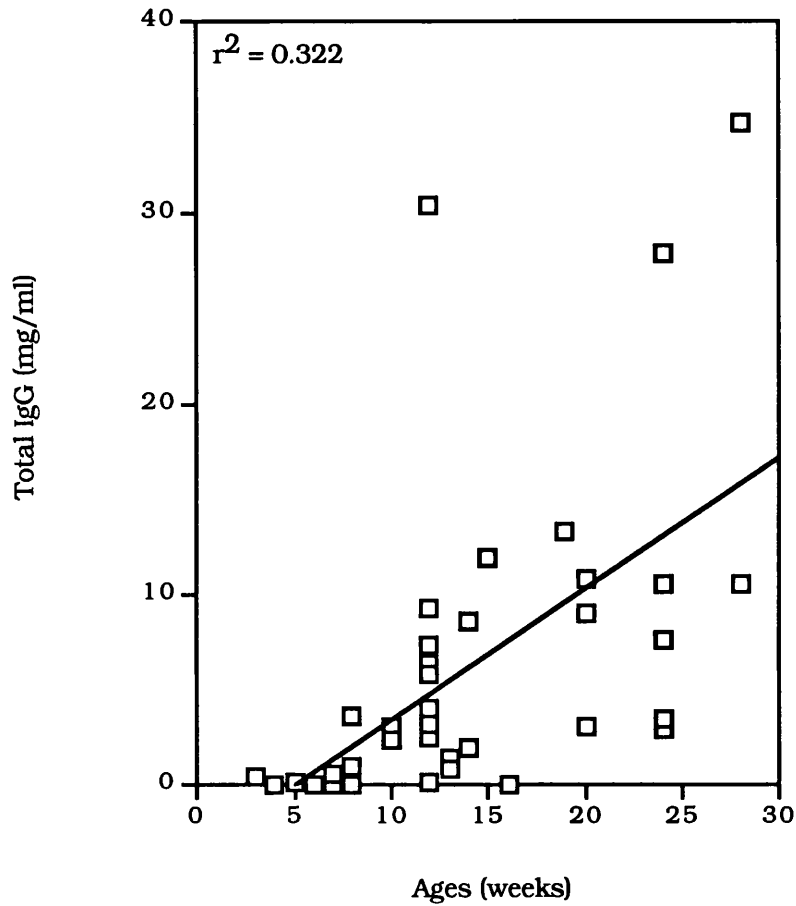
Fig.4.2.4. Total immunoglobulin levels increase with age in Mrl/*lpr* mice, reflecting increased polyclonal B cell activation as well as increases in antigen-driven antibody responses (see chapter 1, section 1.3.5).

4.3. Optimisation and specificity of anti-hsp ELISAs

Ideally an ELISA should be as simple as possible, but with typically heterogeneous ('sticky') sera from MRL/*lpr* mice and human SLE patients non-specific binding causes high backgrounds. Conditions thus have to be established which minimise these effects. In order to define the optimum environment for detection of anti-hsp antibodies, it was necessary to ascertain whether or not antibodies that recognised these proteins were present in sera of MRL/*lpr* mice. A crude experiment was thus set up to determine the existence of such antibodies, and the simple ELISA used in this experiment was based on serial dilutions of protein being matched against serial dilutions of sera, all steps carried out for one hour at 37°C. Despite high backgrounds results obtained in this experiment indicated that there were antibodies present which recognised the coated heat shock protein.

To maximise detection of these antibodies a selection of sera was used in studies to establish optimum conditions for the assay. Sera selected for this study had high anti-dsDNA titres, since if anti-hsp antibodies were involved in the murine disease they would probably be present at relatively high concentrations in sick animals. A variety of serum dilutions were tested to optimise conditions, as well as the presence or absence of several blocking agents, changing incubation periods and temperatures, and examining the effects of different protein coating concentrations (see Table 4.3). The conclusion drawn from these studies indicated that ideal conditions for detection of anti-Hsp90 and anti-Hsp70 antibodies were:

(a)   Coating the relevant heat shock protein overnight at 4°C. There were problems in the initial stages of these experiments with protein stability, (which later transpired to have arisen from source), but once these problems had been resolved the heat shock protein remained stable overnight in BIC at 4°C.

(b)   Use of a blocking agent to fill any vacant sites on ELISA plates. A

variety of agents were examined, and the best results were obtained with

BSA. Similar results were obtained with gelatine, but

this was discarded because of difficulties engendered by the need to

keep the plates warm throughout the experiment .

(c)   Introduction of 1% goat serum into diluted sample sera. This had

the effect of substantially reducing backgrounds, probably by binding

out non-specific antibodies.

(d)   Incubation of the alkaline phosphatase-labelled second antibody

overnight at 4°C.

Anti-Hsp90 Western blotting

Confirmation that antibodies to Hsp90 were present in MRL/*lpr* mice was

achieved by the use of a Western blotting system. Purified Hsp90 and purified

BSA were run on an SDS-PAGE gel and blotted as described previously (chapter

2). Since this technique is far less sensitive than an ELISA system higher

concentrations of sera were required, and it was necessary to pool sera from

more than one animal. Pooled sera from six anti-Hsp90 positive and six anti-

Hsp90 negative MRL/*lpr* mice were thus used to probe for anti-Hsp90

antibodies. As can be seen in Fig.4.3.1., the anti-Hsp90 positive sera bound to

the purified Hsp90 protein but not the BSA, while the anti-Hsp90 negative sera

failed to bind to either protein. It appears therefore that antibodies present in

the pool of positive sera specifically recognise the purified heat shock protein.

Determination of conditions for an anti-hsp ELISA

Parameter	Reagents Used	Temps/Concs	Time
Hsp solvent	PBS, **BIC**	-	-
Hsp concentration	-	0.001-5mg/ml **(0.5mg/ml)**	-
Hsp incubation	-	37°C, **4°C**	1hr, **O/n**
Blocking protein	Gelatine, **BSA**, milk, Casein, goat sera	0.02-5% **(2%)**	-
Blocking incubation	-	**37°C**, 25°C, 4°C	**1hr**, O/n
Pre-incubation with sera	**Goat**, Rabbit sera	37°C, **25°C**, 4°C	**1hr**, O/n
Goat sera dilutions	-	0.02-5% **(1%)**	-
Sample dilutions	PBS, **PBS-T**	1/20-1/10,000 **(1/200)**	-
Sample incubation	-	**37°C**, 25°C, 4°C	**1hr**, O/n
2nd antibody type (IgG)	**Fc**, Fab'	-	-
2nd antibody (Ab) dilutions	**PBS-T**	1/50-1/30,000 **(1/1000)**	-
2nd Ab incubation	-	37°C, 25°C, **4°C**	1hr, **O/n**

Table 4.3. Various parameters were examined during optimisation of the anti-hsp ELISA. The final assay utilised two overnight (O/n) incubations, a blocking protein and addition of goat sera to diluted mouse samples prior to addition to the microtitre plates. Selected parameters are shown in bold type (e.g. BSA was selected as the blocking protein at a concentration of 1%). If no significant difference was evident between parameters the shorter time period or lower concentration was selected.

Antibodies in pooled sera specifically recognise purified Hsp90

90 kd —

A   B   C   D
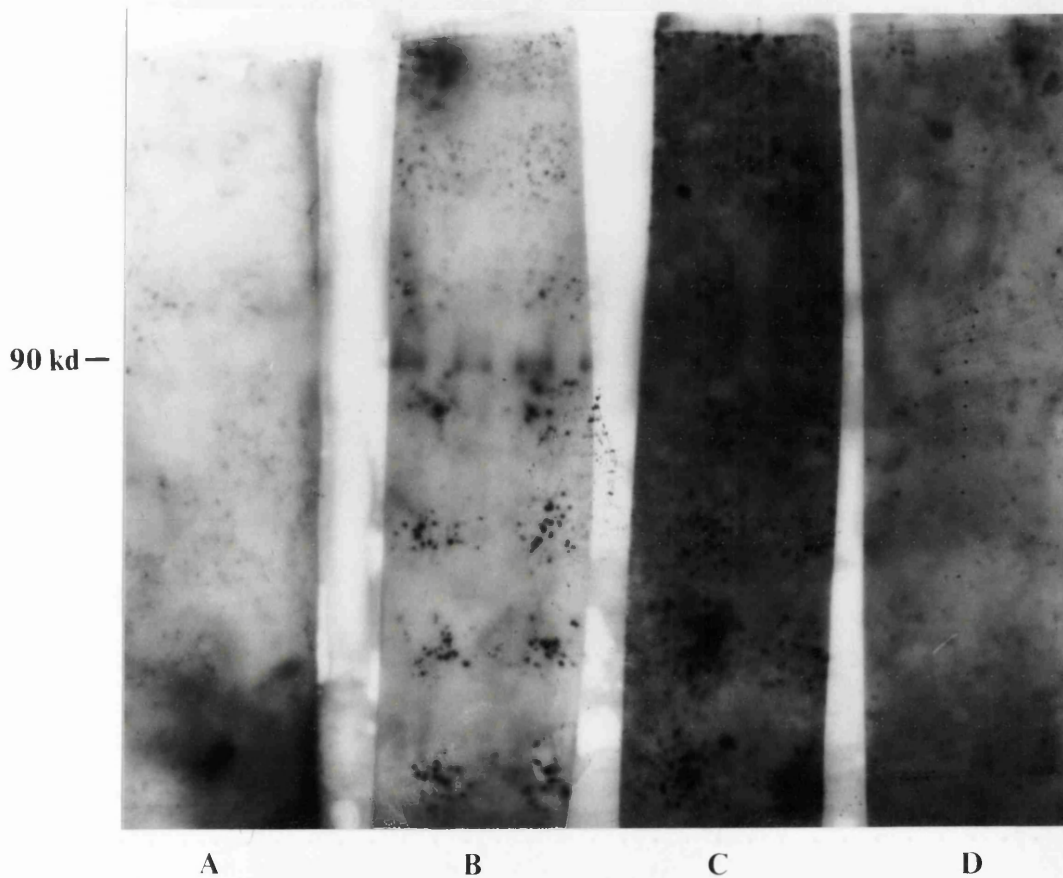
Fig.4.3.1. Antibodies present in MRL/*lpr* sera will bind to purified Hsp90 that has been run on an SDS-PAGE gel (lane B), but not to purified BSA (lane A). Sera from MRL/*lpr* mice that did not have significant levels of antibodies to Hsp90 failed to bind to either the purified heat shock protein (lane D) or BSA (lane C). Note the high levels of non-specific background binding encountered during this experiment which had to be minimised when developing the ELISA system.

128

Competition experiments

Further confirmation that the antibodies in any serum specifically recognise the hsp being examined can be obtained by incubation of the serum with the relevant protein prior to coating on a microtitre plate. This enables antibodies present in serum to bind to the protein in solution, and the effects of competition between coated hsp and hsp in solution should thus result in diminution of the values obtained without pre-incubation with hsp.

In these experiments the relevant heat shock protein was added to the diluted sera of two anti-hsp positive and two anti-hsp negative MRL/*lpr* mice at room temperature at least one hour prior to addition to the microtitre plates. As seen in Fig.4.3.2A, the values obtained from MRL/*lpr* mice positive for anti-Hsp90 antibodies were significantly decreased by prior addition of Hsp90 into the sera, while the anti-Hsp90 negative mice results were virtually unchanged. The diminished values from the antibody positive MRL/*lpr* mice thus appear to be a result of antibodies binding to the hsp in solution, and hence their removal from the pool of antibodies available to bind the coated hsp on the microtitre plate. The amount of competition increased with time, and significantly reduced optical densities (and hence anti-hsp levels) were seen when sera and hsp were left to incubate overnight. Analogous results were observed with anti-Hsp70 antibodies, with a significant reduction in optical densities noted when Hsp70 was incubated with sera prior to addition to the microtitre plates. As with the results from anti-Hsp90 antibodies, the amount of competition increased with time (Fig.4.3.2B).

Competition ELISAs decrease the binding of antibodies to coated hsp on a microtitre plate

A

Anti-Hsp90

Coated protein (µg/ml)

........□........ +ve90

———◇——— +ve90 comp 1hr

— — -⊞- — — +ve90 comp o/n

— — —▲— — — -ve90

- - - -○- - - - -ve90 comp

B

Anti-Hsp70

Coated protein (µg/ml)

———□——— +ve70

........◇........ +ve70 comp 1hr

— — -⊞- — — +ve70 comp o/n

— — —▲— — — -ve70

- - - -○- - - - -ve70 comp
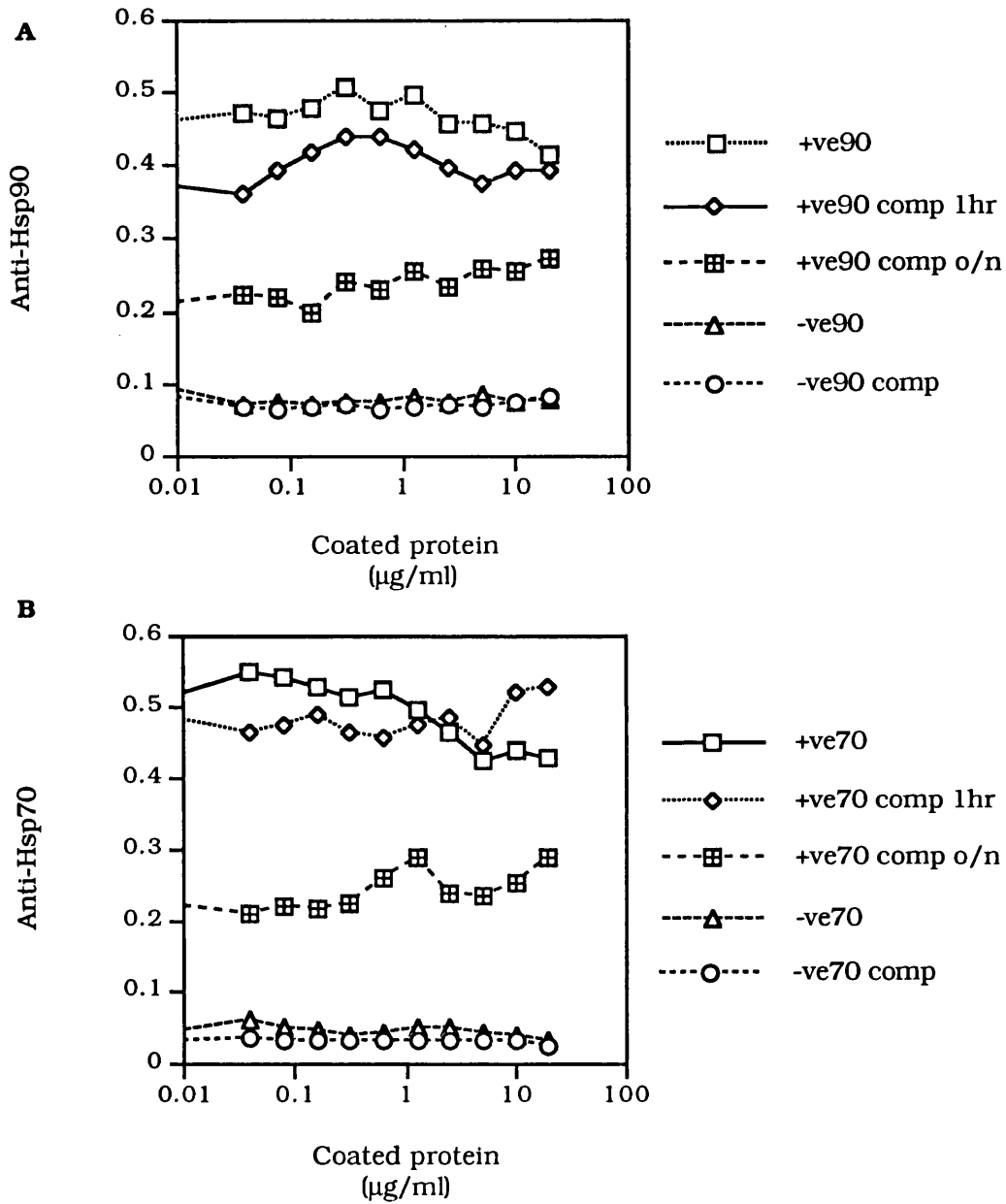
Fig.4.3.2. Antibodies to heat shock proteins present in the sera of diseased MRL/*lpr* mice will bind the heat shock protein in solution, hence decreasing the amount of free antibody available for binding the coated hsps on the plate. A final concentration of 10µg/ml of heat shock protein was added to anti-hsp positive (+ve) or anti-hsp negative (-ve) sera diluted 1;100 in PBS-Tween20 and the sera/hsp mixture incubated at room temperature for 1 hour (1 hr) or overnight (o/n). For both Hsp90 (A) and Hsp70 (B), competitive binding (comp) of antibodies increases with time. The Y-axis units are optical density values that have been equalised for interplate variation using the values given by a control serum on each plate.

4.4. Detection of anti-Hsp90 antibodies

Having established there were antibodies in sera of MRL/*lpr* mice that recognised Hsp90, an investigation of MRL/*lpr*, MRL/++ and Balb/c mice was undertaken to determine what relevance to the disease state these antibodies might have. Sera were obtained from mice over a range of ages, and anti-Hsp90 IgG antibody levels determined by ELISA. The results, depicted in Fig.4.4.1., show that 45% (20/44) of the MRL/*lpr* mice surveyed had antibody levels that were greater than twice the mean plus two standard deviations (SD) levels found in 46 control Balb/c mice. Furthermore, if hsp values for MRL/*lpr* mice younger than 12 weeks of age were disregarded in order to more fully examine the link between mice with active disease and anti-Hsp90 antibody levels, the percentage of anti-Hsp90 antibody positive mice exceeded 60% (19/30). Serological abnormalities in these autoimmune mice become evident from around 12 weeks (see Fig.4.2.1B), and it is interesting to note that only 1/15 MRL/*lpr* mice younger than this had elevated IgG antibody levels.

Plotting these results against age of the mice (Fig.4.4.2) reveals an increase in anti-Hsp90 antibody levels with age and hence disease. An exponential curve is shown, since the increase in antibody levels appears to be better defined as exponential ($r^2 = 0.302$) than linear ($r^2 = 0.186$). These results were corroborated by the use of sera obtained from a second population of mice, which were maintained in the laboratory of a project collaborator in Philadelphia, USA. Sera from mice of three different ages (6, 12 and 28 weeks) were extracted from this population and sent to our laboratory for examination. Results obtained from these mice are also included in Fig.4.4.2., and provide supporting evidence for an increase in anti-Hsp90 antibodies with onset of disease.

The elevated titres of antibodies evident in MRL/++ mice show an intriguing pattern, with some similarity to data from MRL/*lpr* mice. However, onset of production of antibodies to Hsp90 appears somewhat delayed in these mice,

131

supporting the notion that these antibodies may have a role in the disease process. The high anti-Hsp90 antibody value obtained from one young MRL/++ mouse distorts the results somewhat, and if this result is disregarded the relationship between age and antibody levels becomes significant (r^2= 0.352). This mouse was later shown to have high anti-ssDNA and proteinuria levels and may therefore have developed an unusually rapid onset of disease, not normally observed in these mice. In contrast, Balb/c mice showed no such increase in antibody levels with increasing age, remaining at a uniformly low level.

Anti-Hsp90 in kidney eluates

Kidney eluates from a small number of MRL/*lpr* mice were examined for the presence of anti-Hsp90 antibodies. Antibodies were not detected in eluates from six five-month old mice tested.

Significantly elevated levels of antibodies to Hsp90 are found in MRL/*lpr* and MRL/++ mice compared with Balb/c controls
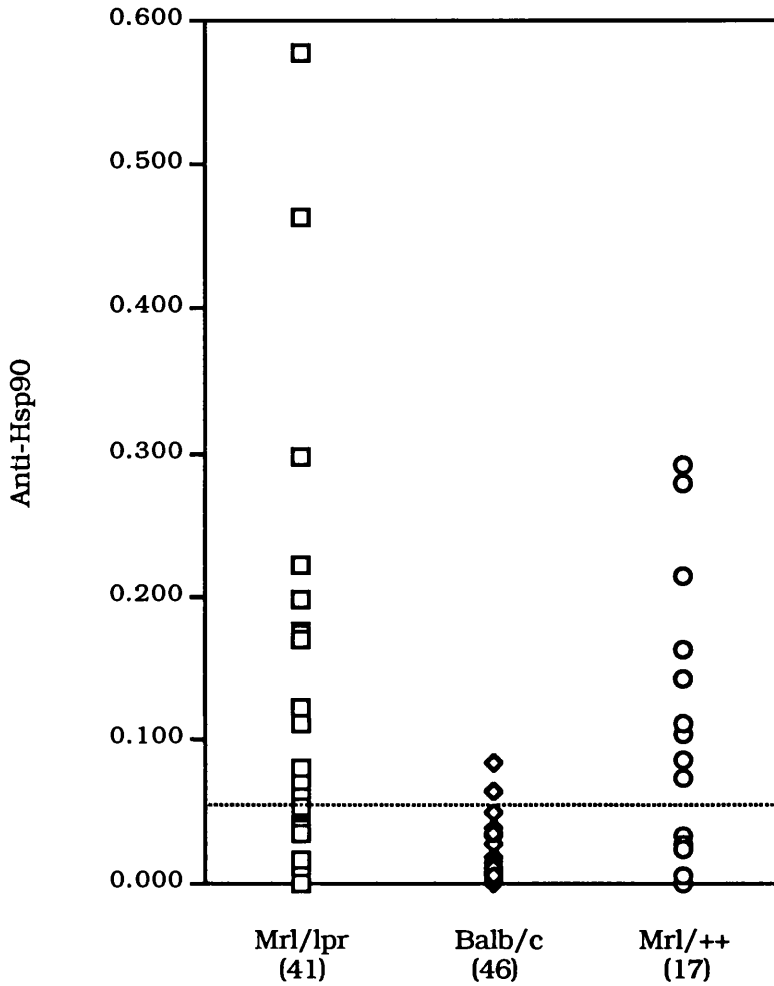
Fig.4.4.1. Antibodies to Hsp90 are common in both MRL/*lpr* and MRL/++ mice, and are found in significantly elevated concentrations compared with levels observed in control Balb/c mice. The dotted line indicates the mean + 2SD of Balb/c Hsp90 levels (n=46). Nearly 50% of the MRL/*lpr* mice studied had levels above this value (20/41) and a similar percentage was found in MRL/++ mice (9/17).

Anti-Hsp90 antibody levels increase with age in MRL/*lpr* mice
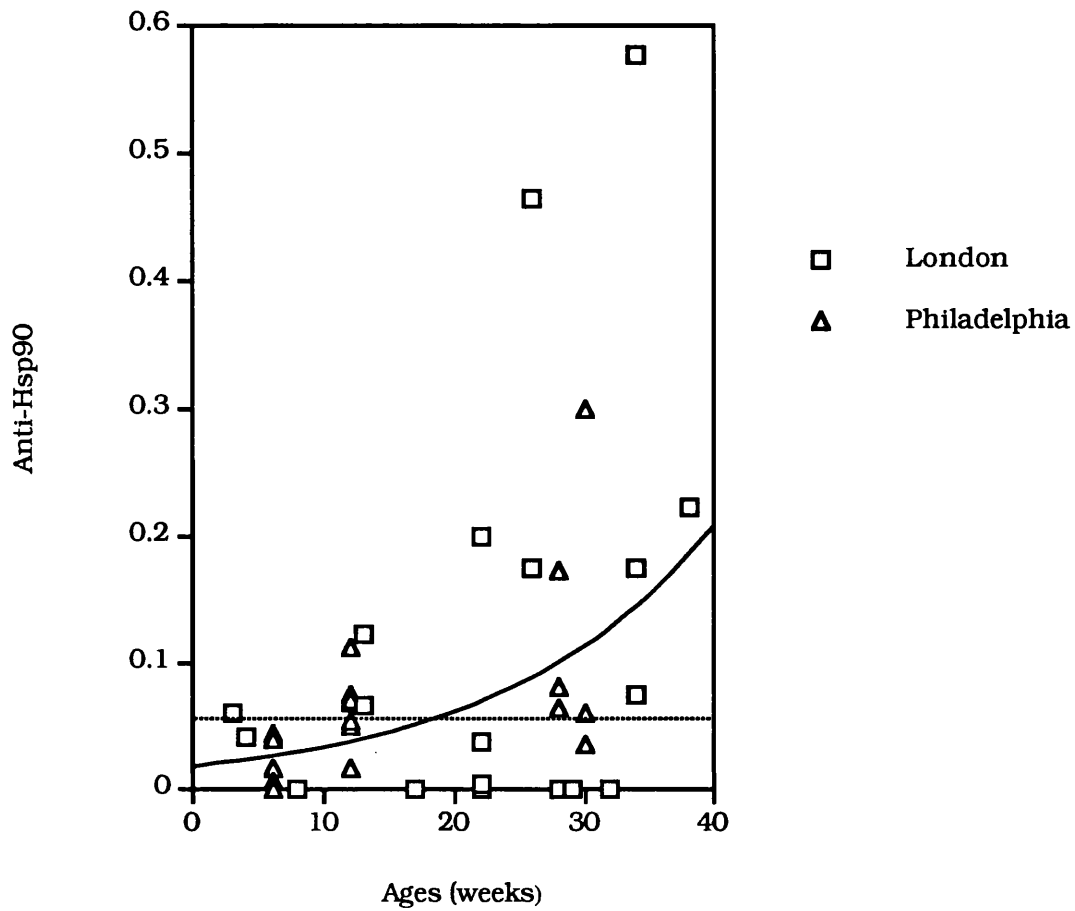
Fig.4.4.2. Data from two populations of MRL/*lpr* mice, showing that anti-Hsp90 antibodies develop in the majority of MRL/*lpr* mice over twelve weeks of age. The antibody concentrations show an increase with age. The dotted line represents the mean anti-Hsp90 value + 2 SD of the Balb/c control mice.

Antibodies to Hsp90 increase with age in MRL/++ mice, but not Balb/c mice
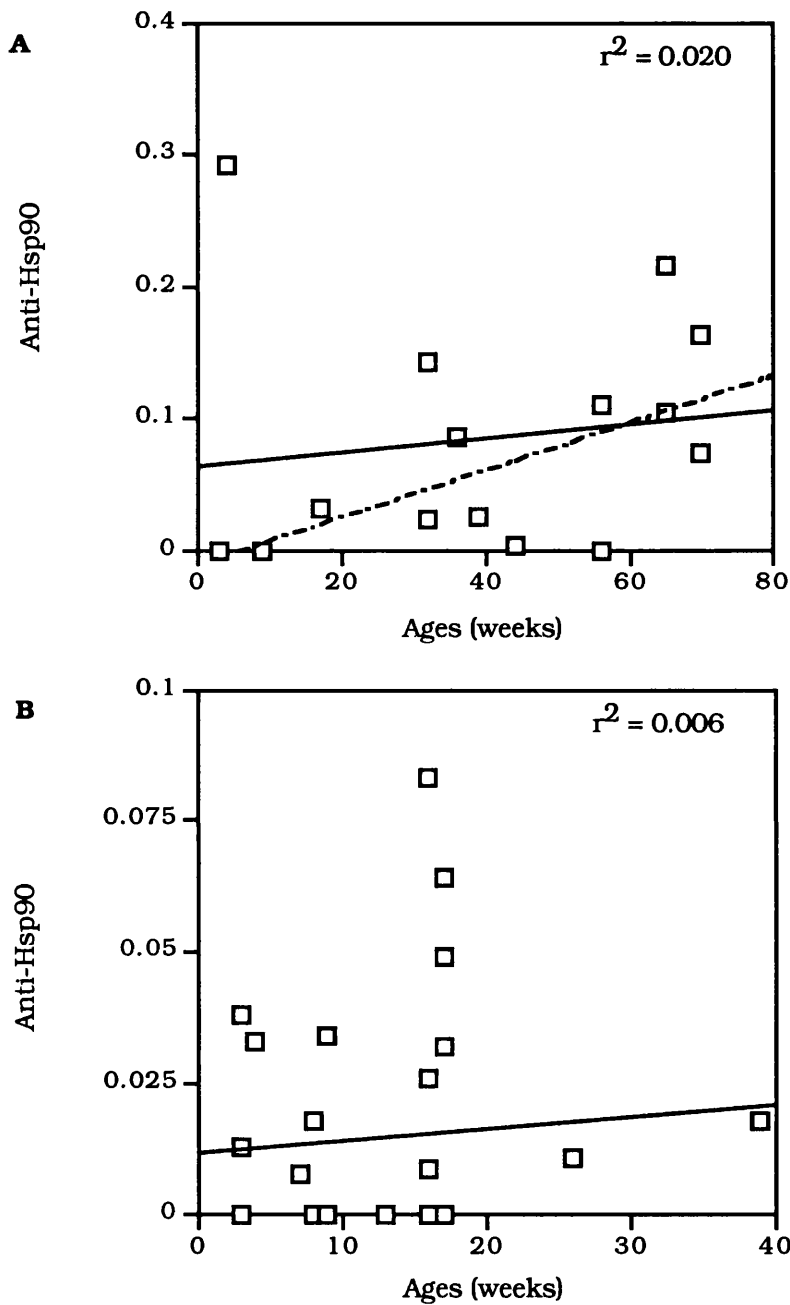
Fig.4.4.3. (A) Antibodies to Hsp90 increase with age in MRL/++ mice, but not in Balb/c mice (B) although their appearance is somewhat retarded in comparison with generation of these antibodies in MRL/lpr mice (Note that the X-axis timescale used with MRL/++ mice is approximately double that for Balb/c and MRL/lpr mice). There is no statistical correlation with age in the MRL/++ study, although by discarding the result from the anomalous young mouse with high anti-Hsp90 levels good correlation is observed (Represented by dotted line. r^2 = 0.356; refer text)

There is no correlation between Anti-Hsp90 levels and anti-DNA levels in MRL/*lpr* mice
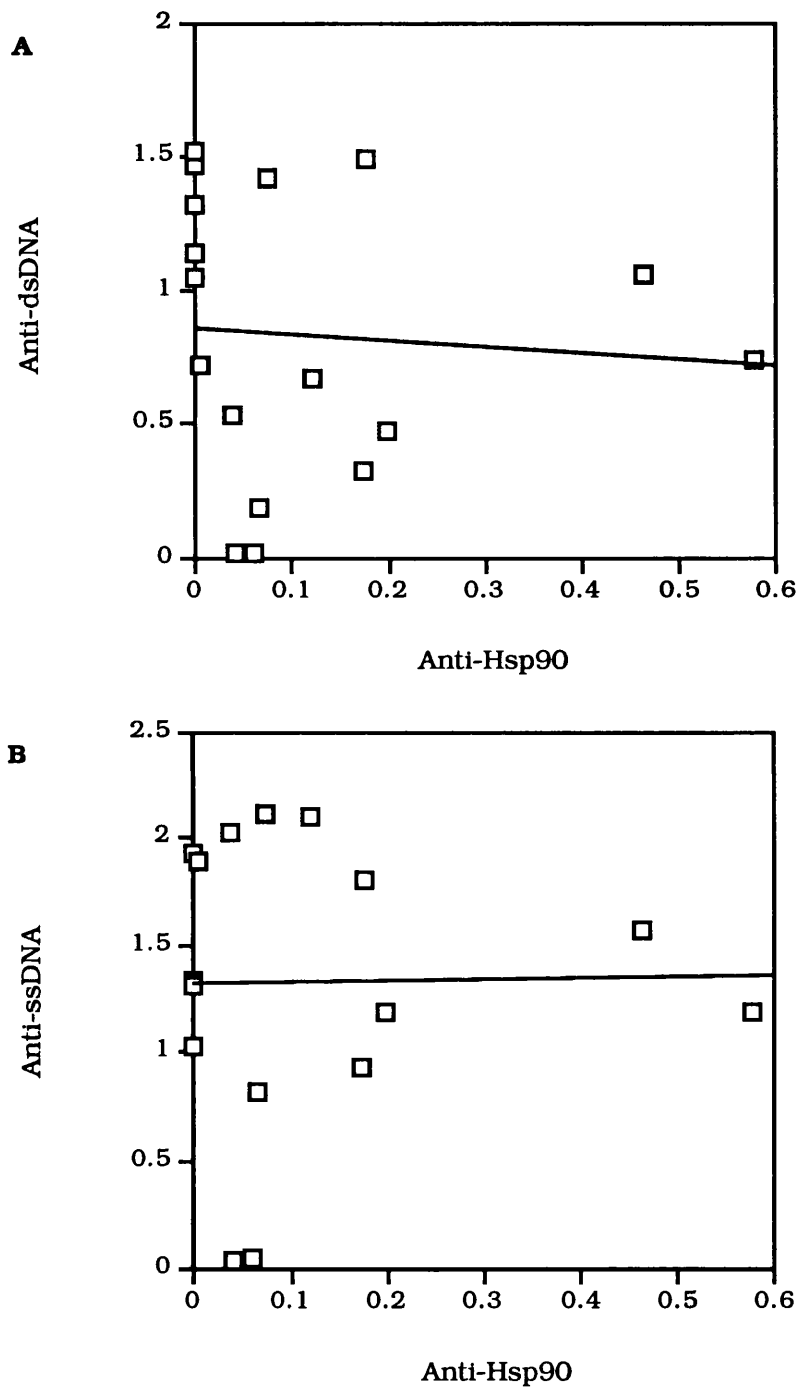
Fig.4.4.4. Levels of antibodies to Hsp90 in MRL/*lpr* mice show no correlation with levels of anti-ssDNA (A) or anti-dsDNA (B) antibodies.

Anti-Hsp90 antibodies in development

Sera were extracted from two MRL/*lpr* and two Balb/c mice at 5, 6. 7, 8, 10, 12, and 14 weeks in order to chart development of anti-Hsp90 antibodies in the autoimmune mouse (Fig.4.4.5). The titres obtained from these mice are below the mean + 2SD of the control Balb/c population, but there appears to be some evidence for early generation of a response to Hsp90 and anti-ssDNA. One of the MRL/*lpr* mice appeared to develop antibodies to the heat shock protein from as young as five weeks of age, a rise mirrored by generation of anti-ssDNA, but not anti-dsDNA, antibodies. The other MRL/*lpr* mouse did not develop anti-Hsp90 antibodies until 14 weeks, although a similar delay in development of anti-ssDNA and anti-dsDNA antibodies was evident in this mouse. In contrast, neither of the Balb/c mice generated positive responses to these antigens. Thus, there appears to be a link between production of antibodies to Hsp90 and onset of other serological manifestations of the disease.

Anti-Hsp90 IgM antibodies

Production of IgM antibodies to Hsp90 was also observed in MRL/*lpr* mice, and increased antibody levels were found in 37% (7/19) of the autoimmune mice compared with Balb/c controls, with the mean anti-Hsp90 value for MRL/*lpr* mice of 0.046 over five-fold the mean value for Balb/c mice (0.0084), and nearly three times the mean value obtained from MRL/++ mice (0.016). The levels of these antibodies bears little relationship to age (Fig.4.4.6), although elevated antibody levels appeared to occur primarily in younger animals.

The age-matched young mice from 5-14 weeks of age were tested for IgM antibody levels, but these were not detected in either young MRL/*lpr* mice or Balb/c controls.

Antibodies to Hsp90 are detected in young MRL/*lpr* mice

A

Balb/c 1
Balb/c 2
Lpr 1
Lpr 2

Ages (weeks)

B

Lpr 1 ds
Lpr 2 ds
Lpr 1 ss
Lpr 2 ss

Ages (weeks)
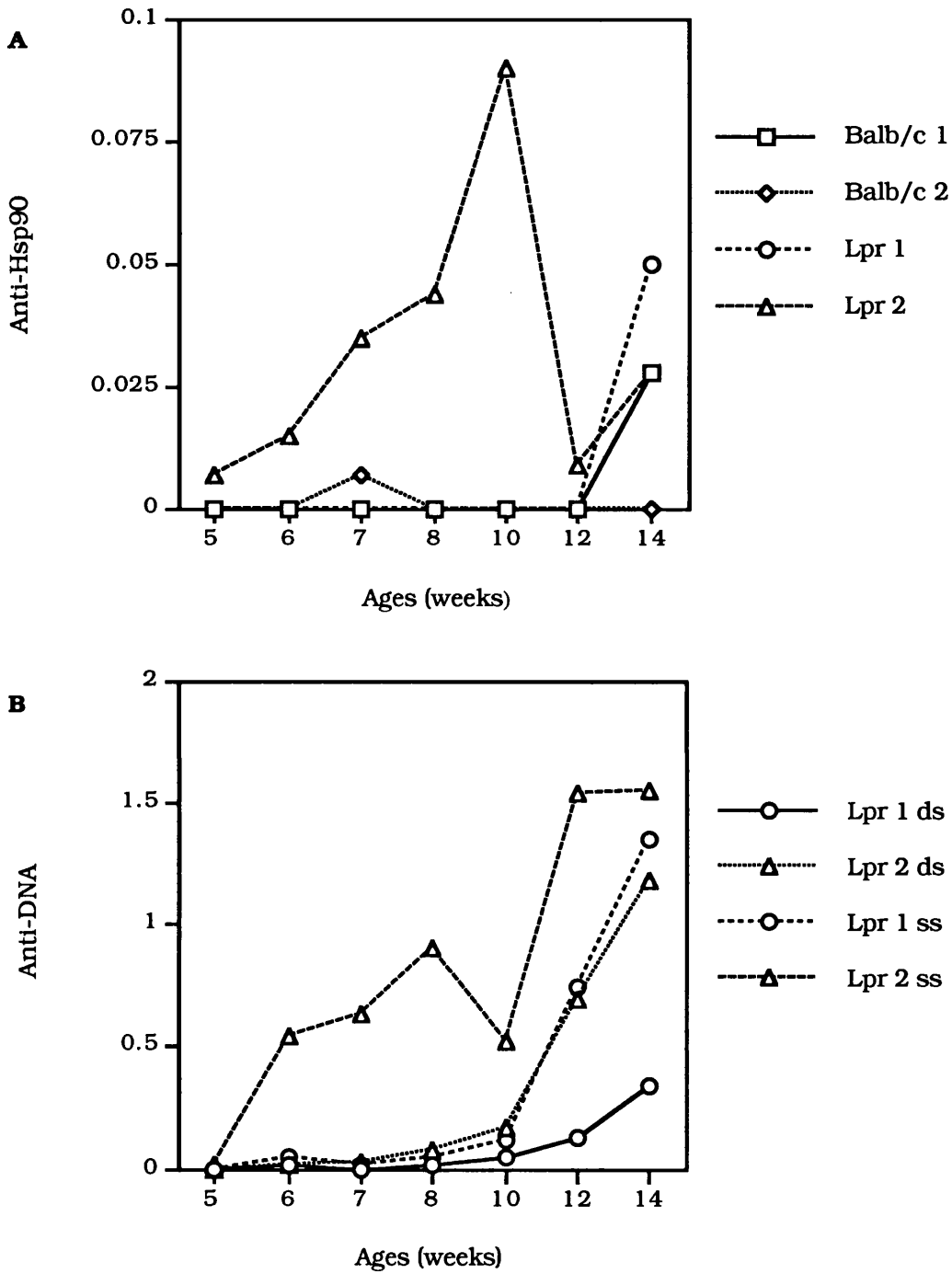
Fig.4.4.5. (A) Antibodies to Hsp90 can be observed in MRL/*lpr* mice as young as six weeks of age. Anti-ssDNA antibodies are detected at a similar time, although appearance of anti-Hsp90 antibodies precedes development of anti-dsDNA antibodies (B).

Elevated levels of IgM antibodies are also found in MRL/*lpr* mice compared with control mice
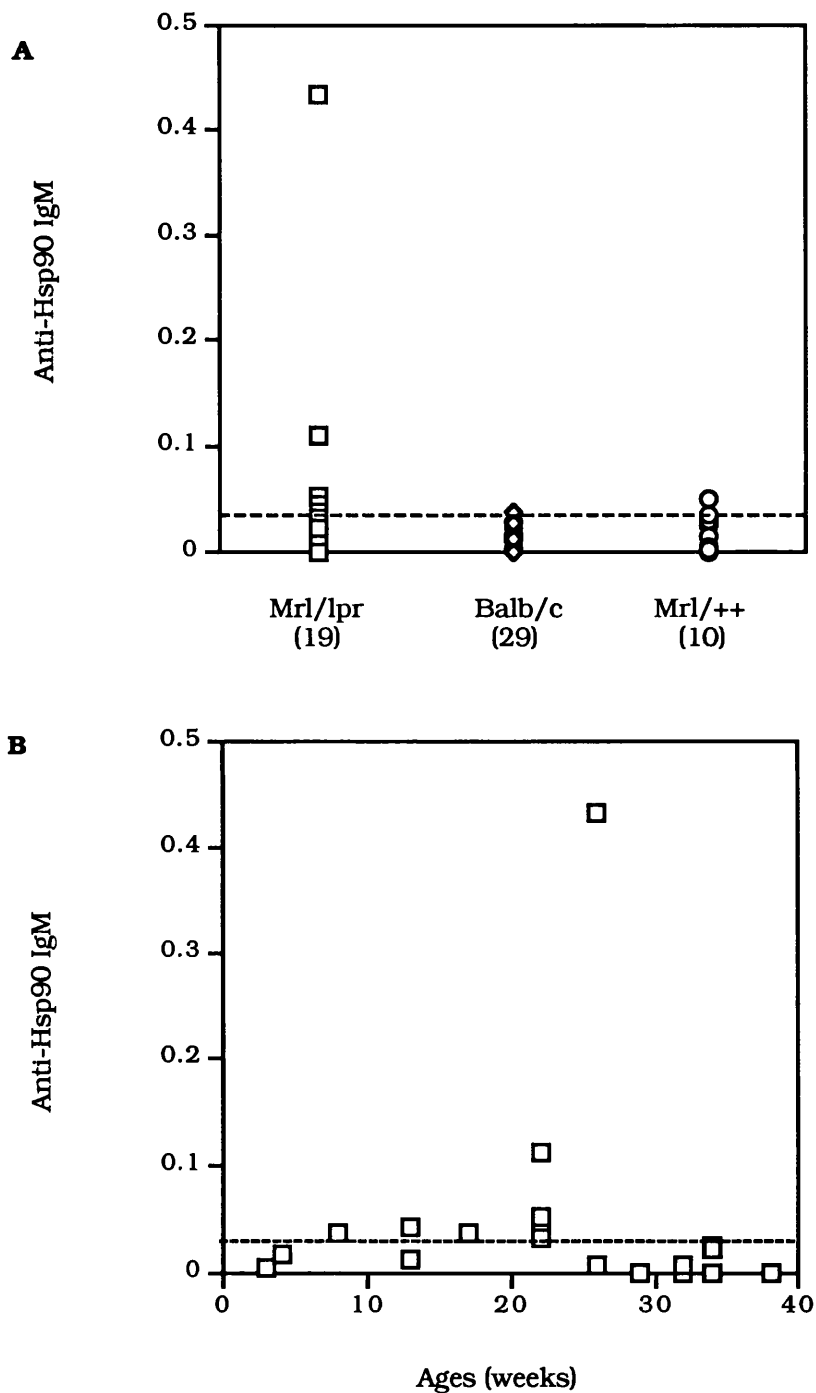
Fig.4.4.6. Elevated IgM antibodies to Hsp90 are found in a subset of MRL/*lpr* mice compared with Balb/c and MRL/++ mice (A) although there is no clear relationship with age (B).

Anti-Hsp90 IgM and IgG antibodies are correlated in MRL/*lpr* mice
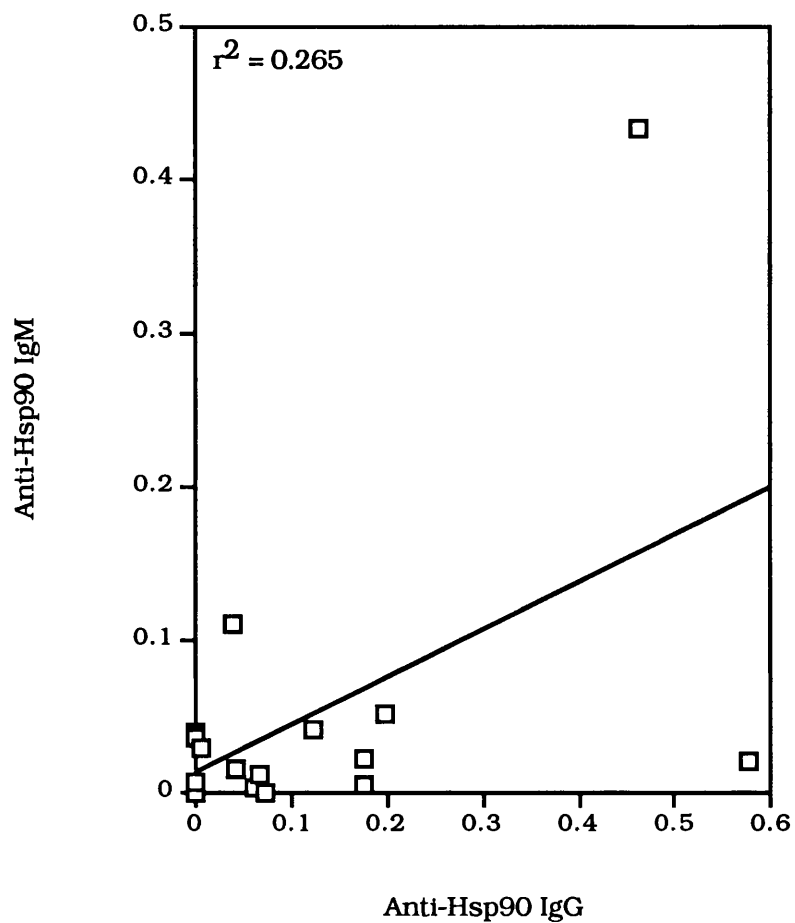
Fig.4.4.7. Anti-Hsp90 IgM antibody levels are correlated with anti-Hsp90 IgM levels in MRL/*lpr* mice (p= 0.029).

140

4.5. Detection of anti-Hsp70 and anti-Hsp60 antibodies

Anti-Hsp70 antibodies

Non-specific elevation of Hsp72 protein observed in the MRL/*lpr* mouse (see section 3.3) focused attention on the need to test these mice for presence of antibodies to this protein. In the absence of an ideal commercially available antigen, human Hsp70 (Stressgen) was used to coat microtitre wells for these ELISAs. This is a mixture of Hsp70 proteins, comprising mostly hsc70 (Hsp73), the constitutively expressed member of the Hsp70 family. However, a small amount of the inducible member of the Hsp70 family, Hsp72, can also be found in the mixture and for this reason antibodies found in this study are referred to as anti-Hsp70 rather than anti-Hsp73 or anti-Hsp72.

MRL/*lpr* mice had significantly increased levels of antibodies to Hsp70 compared with Balb/c mice, and over 80% (19/23) of these mice had antibody levels greater than the mean + 2SD of the levels found in control mice (Fig.4.5.1). As with anti-Hsp90 antibody titres there was an increase in levels with age. Production of anti-Hsp70 antibodies was significantly elevated from twelve weeks in development compared with Balb/c controls (Fig.4.5.2A). MRL/++ mice also showed a response to Hsp70 which mirrored that seen with Hsp90. There was significant elevation in levels of antibodies to the hsp detected by ELISA, these antibodies first appearing at significant levels from about 30 weeks (Fig.4.5.2B).

Anti-Hsp70 antibody levels were plotted against other parameters to examine correlations (Fig.4.5.3). However, anti-Hsp70 levels showed no correlation with total IgG levels, anti-Hsp90, anti-ssDNA, or anti-dsDNA levels.

Antibodies to Hsp70 were detected in young MRL/*lpr* mice, and seemed to show a typical antibody response (Fig.4.5.4). No such response was noted in the two Balb/c mice examined.

<u>Anti-Hsp60 antibodies</u>

A significant proportion of MRL/*lpr* mice were also shown to have antibodies to Hsp60. Approximately 37% of these mice (10/27) had elevated levels compared with Balb/c controls (Fig.4.5.5A). The levels of these antibodies were significantly correlated with anti-Hsp70 levels (Fig.4.5.5B; p = 0.002), but as with anti-Hsp70 antibodies no correlations were seen with anti-Hsp90, anti-ssDNA, anti-dsDNA or total IgG levels. Interestingly, similar correlations between anti-Hsp70 and anti-Hsp60 antibody levels in MRL/++ mice were observed (Fig.4.5.6A). Furthermore, antibodies to these two hsps also showed significant correlation with anti-Hsp90 antibody levels in these mice (Fig.4.5.6B and C).

Antibodies to Hsp70 are found at significantly higher levels in MRL/*lpr* mice compared with control mice
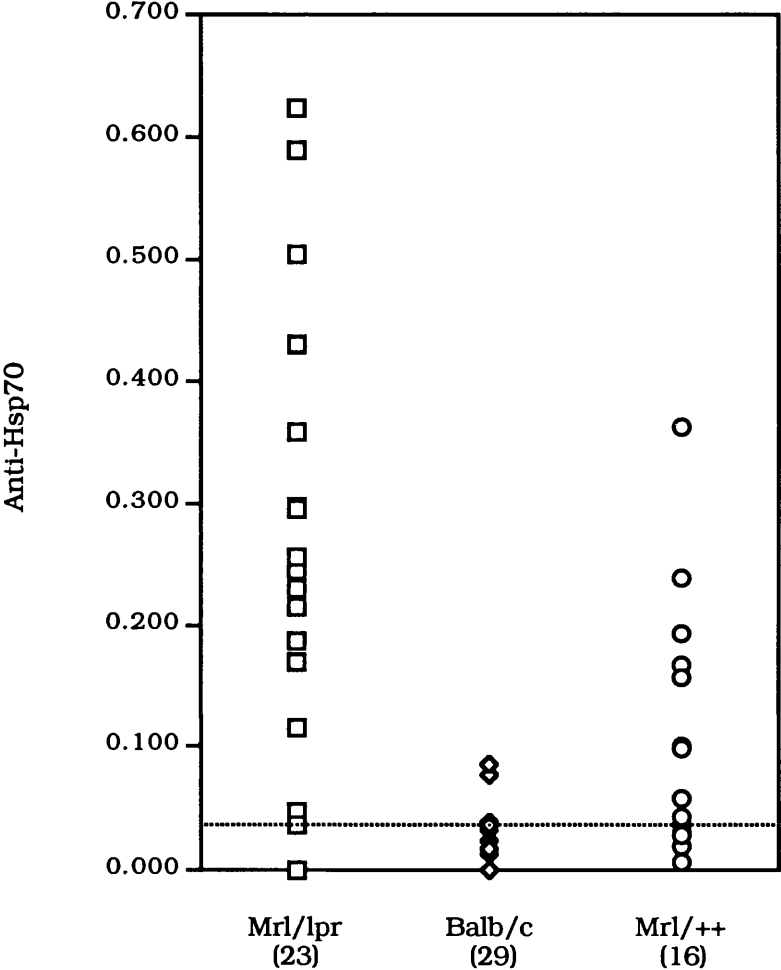
Fig.4.5.1. Nearly 80% of MRL/*lpr* mice have anti-Hsp70 antibody levels greater than the mean + 2SD of Balb/c mice (represented by the dotted line). Similar elevation is observed in samples obtained from MRL/++ mice.

Levels of anti-Hsp70 antibodies in MRL/*lpr* mice rise with age

A

$r^2 = 0.218$

(Scatter plot: x-axis "Ages (weeks)" from 0 to 40; y-axis "Anti-Hsp70" from 0 to 0.7)

B

(Scatter plot: x-axis "Ages (weeks)" from 0 to 80; y-axis "Anti-Hsp70" from 0 to 0.4)
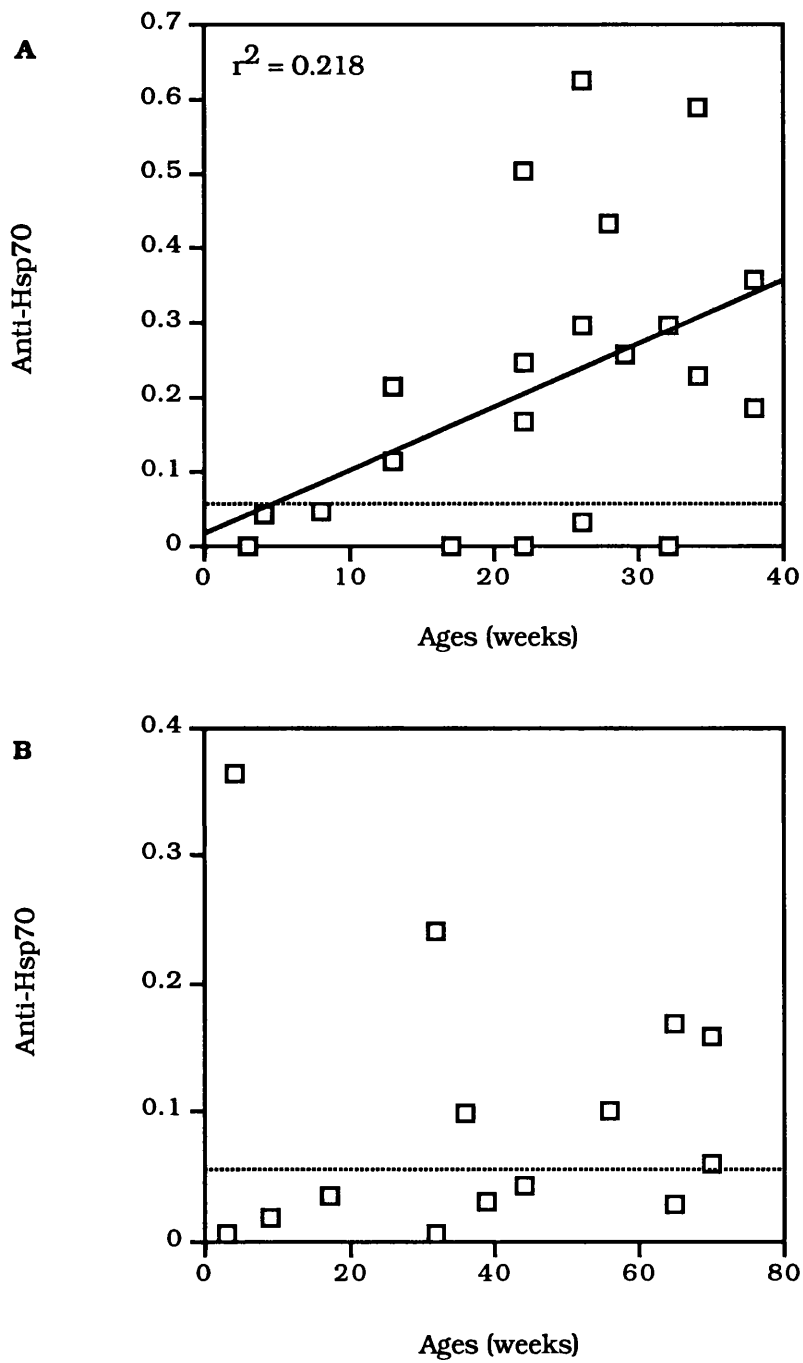
Fig.4.5.2. (A) Antibodies to Hsp70 increase with age in MRL/*lpr* mice, while a similar pattern is observed later in development in MRL/++ mice (B), although this fails to reach statistical significance.

Anti-Hsp70 antibody levels in MRL/*lpr* mice are not correlated with anti-Hsp90, anti-ssDNA, anti-dsDNA or total IgG
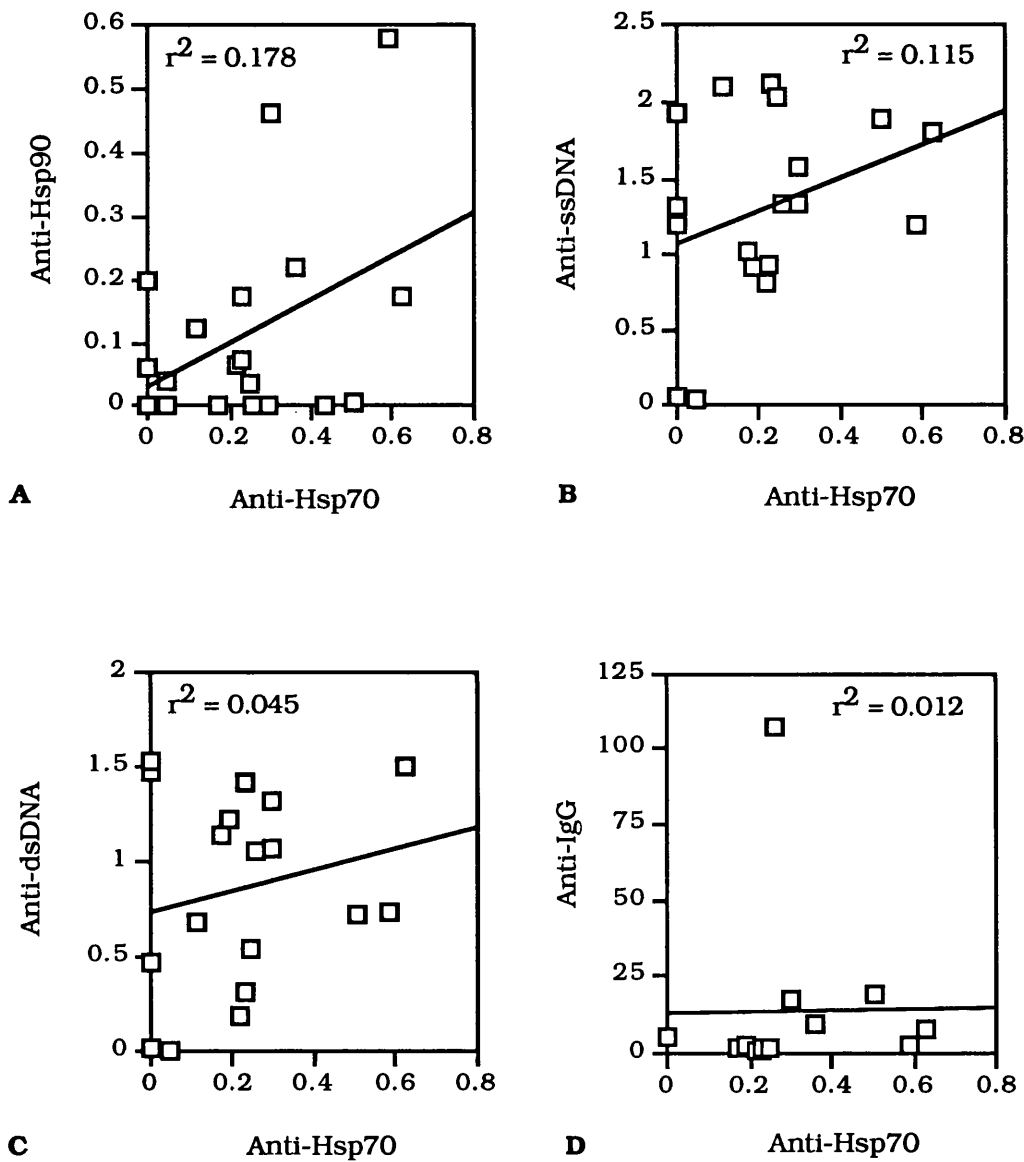
Fig.4.5.3. Anti-Hsp70 antibody levels in MRL/*lpr* mice show no correlation with many other serological features of MRL/*lpr* disease, including levels of antibodies to Hsp90 (A), ssDNA (B), dsDNA (C) or total IgG levels (D).

Anti-Hsp70 antibodies are found at elevated levels in young MRL/*lpr* mice
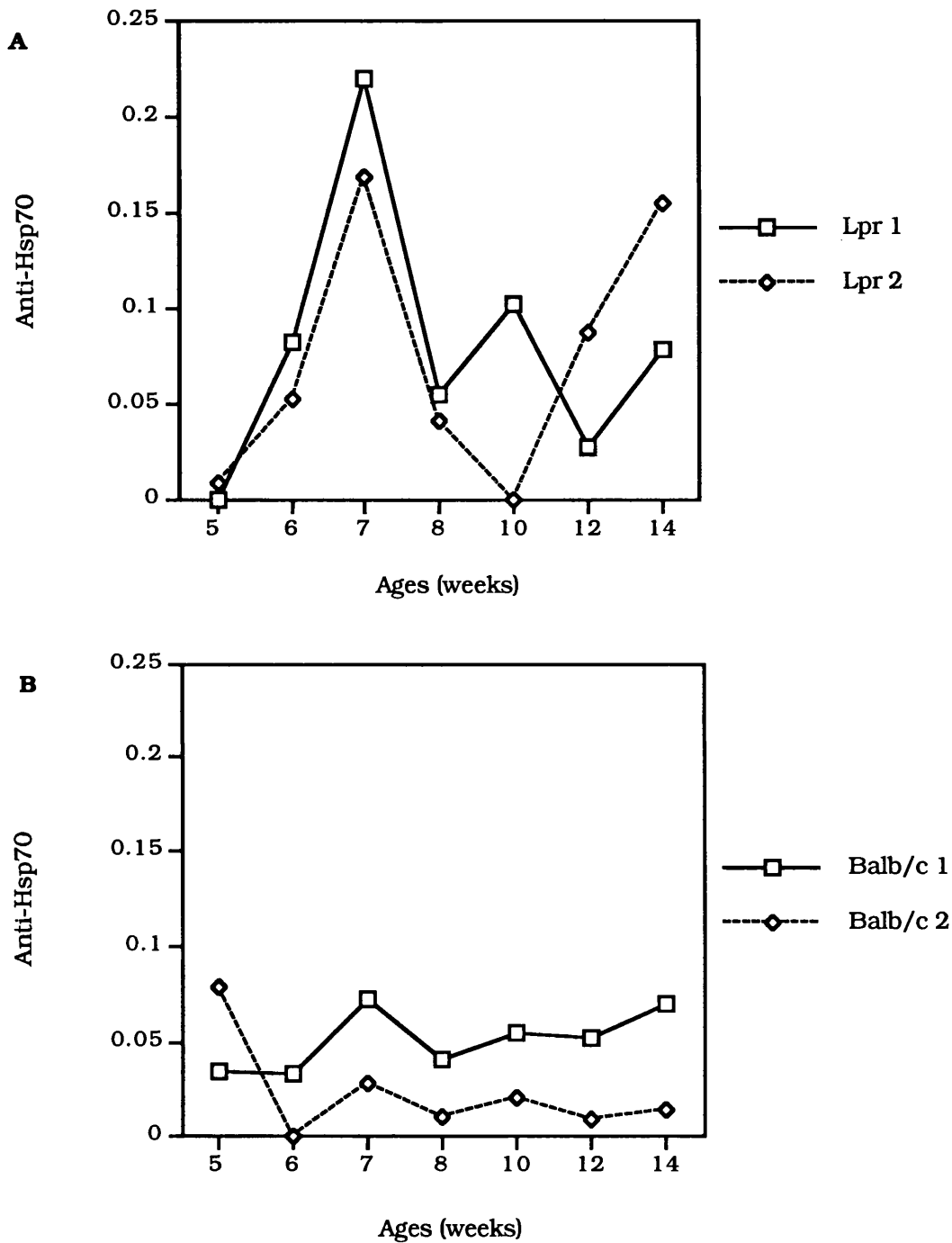
Fig.4.5.4. An immune response to Hsp70 appears to be a feature of young MRL/*lpr* mice, and this is apparent from six weeks in the two MRL/*lpr* examined (A). However, no such response is noted in young Balb/c mice (B).

A tight correlation is seen between antibodies to Hsp60 and Hsp70
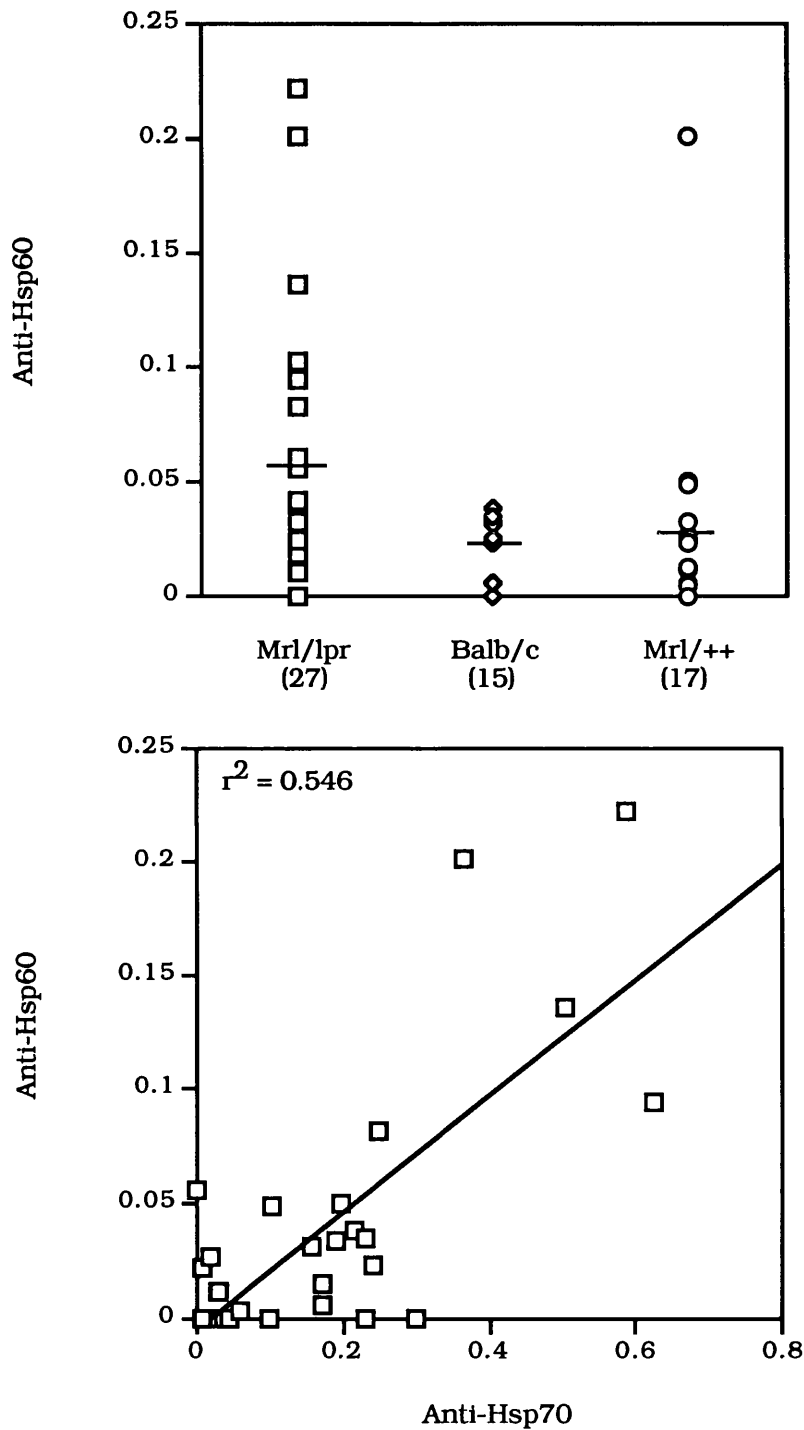
Fig.4.5.5. (A) Elevated levels of antibodies to Hsp60 are also seen in a proportion of MRL/*lpr* and MRL/++ mice compared with Balb/c controls. (B) The levels of antibodies to Hsp60 and Hsp70 are significantly correlated in MRL/*lpr* mice (p = 0.002).

Anti-Hsp60, Anti-Hsp70 and Anti-Hsp90 levels are correlated in MRL/++ mice
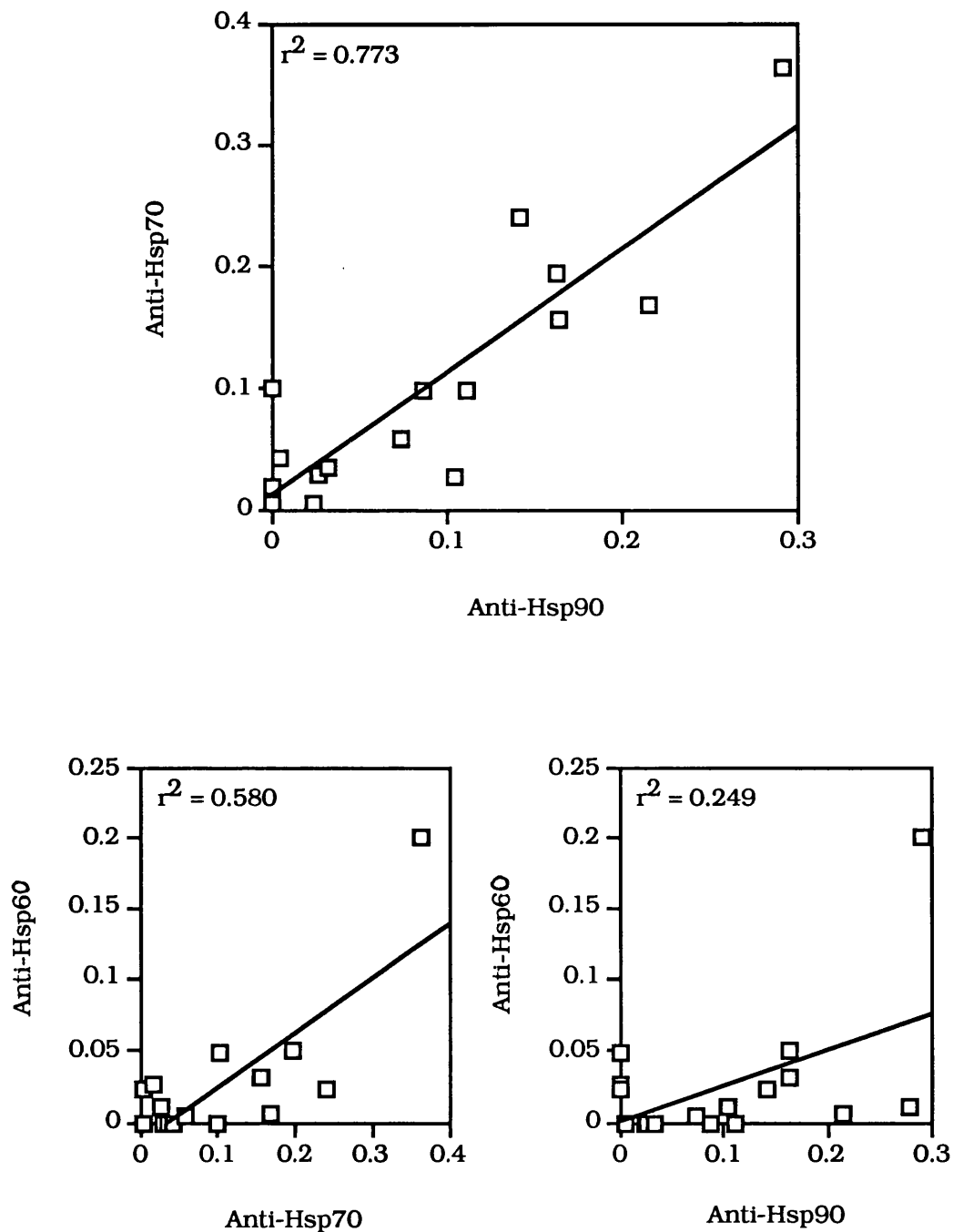
Fig.4.5.6. Levels of antibodies to all three hsps - Hsp90, Hsp70 and Hsp60 - show significant degrees of correlation in MRL/++ mice. Particularly strong correlations were found between anti-Hsp90 and anti-Hsp70 levels (p < 0.001) and anti-Hsp70 and anti-Hsp65 (p = 0.001), while a less strong correlation was noted between anti-Hsp90 and anti-Hsp65 (p = 0.042).

CHAPTER 5: HSP90 PROMOTER IN CELL LINES

T cells, B lymphocytes and monocytes/macrophages are three abundant types of cell in the spleen, and evidence has been presented to suggest that these cells may have intrinsic defects in MRL/*lpr* mice. In addition, there are a number of unusual cells in the spleens of MRL/*lpr* mice, including the CD4⁻CD8⁻ double negative T cell. To further investigate the role of Hsp90 in MRL/*lpr* disease three cell lines were used, representing the aforementioned cell types. The T cell line chosen for these experiments was Jurkat E6, derived from a human T cell leukaemia (Weiss *et al.*, 1984). The cell line selected to represent B lymphocytes was the Burkitt lymphoma-derived Daudi cell (Klein *et al.*, 1968) and the monocytic-like line selected was U-937 (Sundström and Nilsson, 1976).

Cells were transfected with four plasmid constructs of the Hsp90 promoter, as well as a control plasmid, IE, containing a herpes simplex virus immediate early gene promoter. In this manner it is possible to investigate the effect of stress on the mechanism of expression of Hsp90 in these cell types, and thus may provide a clue to the elevated expression of the heat shock protein observed in human SLE patients and MRL/*lpr* mice.

The four Hsp90 promoter constructs used in these experiments were derived from the human Hsp90β gene (see Fig.2.2.2). Construct A includes three promoter HSEs, with the CAT gene spliced into the first exon of the Hsp90 gene. Construct B and construct D use a cryptic splice site from the middle of the first intron to express the CAT gene, B including both promoter and intron HSEs, while D having only the intron HSEs. Construct C does not contain any recognised HSEs and is reputed to be unresponsive to heat shock, although the construct remains capable of driving CAT gene expression. The CAT gene has been spliced into the first exon of the HSp90β gene in construct C, as with construct A.

5.1. Daudi cells

The Hsp90 constructs responded to heat shock in the most predictable way in the Daudi cell line. In this instance the IE control and the HSE-less C construct showed slightly decreased expression after heat shock, while the three HSE-containing constructs, A, B and D, increased expression two-fold to four-fold over basal levels. Interestingly, the B construct, with HSEs in the promoter region as well as in the first intron of the Hsp90β gene, was induced to a lesser degree than either the A construct (with only the promoter HSEs) or the D construct (with only the intronic HSEs). The basal expression of each construct is interesting. It was noted that the basal levels of expression of the Hsp90 constructs were much lower than the IE construct expression in all three cell lines, and in Daudi cells basal expression levels of these constructs was only one-fifth that of the IE construct. Surprisingly, the C construct was expressed at the highest levels in the cells prior to heat shock, and the B construct had the second highest levels of expression.

5.2. U937 cells

Of the three cell lines examined the four Hsp90 constructs respond most strongly to heat shock in U937 cells, which show some characteristics of monocytes. The basal level of expression of these constructs was very low in these cells, although the levels of expression of the IE construct were also reduced in comparison with the other cell lines. The heat shock induction of these constructs was in the order of 20-fold, suggesting a rapid and strong response to stress in these cells. A surprising feature was the induction of the C construct, which does not contain HSEs. The obvious explanation for this would be contamination with another construct, but when the construct was checked this was ruled out as a possibility. In addition, the control IE construct also

increased expression after heat shock. The mechanism responsible for induction of these construct thus remains unknown, as does the mechanism for the induction of all the Hsp90 constructs, since the order of increased expression is approximately equivalent in all of them. However, the induction of these constructs was much greater than that of the IE construct, and it is reasonable to assume that this phenomenon is linked to a feature common to all four Hsp90 promoter constructs and is not a non-specific response to heat stress in these cells. There may be, for example, a non-characterised HSE present in the Hsp90 constructs.

5.3. Jurkat cells

The expression patterns of the various cell lines in the T cell Jurkat line after heat shock were similar to those observed in Daudi cells, with the notable exception of the full-length B construct, which along with the C construct and the IE control actually showed decreased expression after heat shock. The D construct was the most highly induced of the constructs, increasing expression approximately three-fold over basal levels, while the A construct doubled its expression after heat shock. Why the B construct should decrease expression after heat shock is perplexing. Basal levels in these cell lines were interesting, with the B and D constructs having markedly increased expression compared with the A and C constructs,. This suggests the intronic HSEs may be important in the expression of Hsp90 in this cell type, since this feature distinguishes B and D from the other two constructs.

Daudi cells show expected expression of Hsp90 constructs
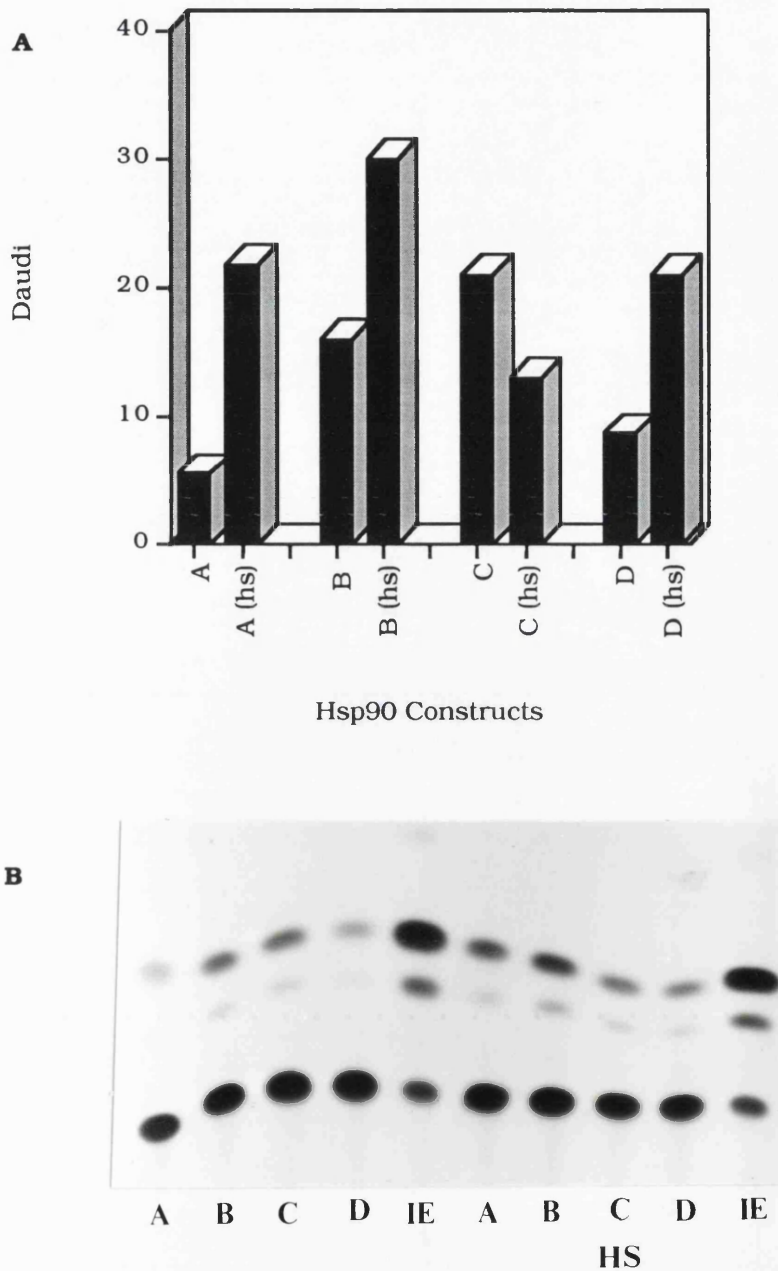
Fig.5.1.1. Daudi cells show a more predictable pattern of expression on heat shock than U937 cells, with a decrease in the HSE-less C construct expression and increased expression of the three HSE-containing constructs. The IE construct is not shown on this graph, since expression levels were an order of magnitude greater than the Hsp90 constructs.

U937 cells markedly increase expression of Hsp90 in heat shock conditions
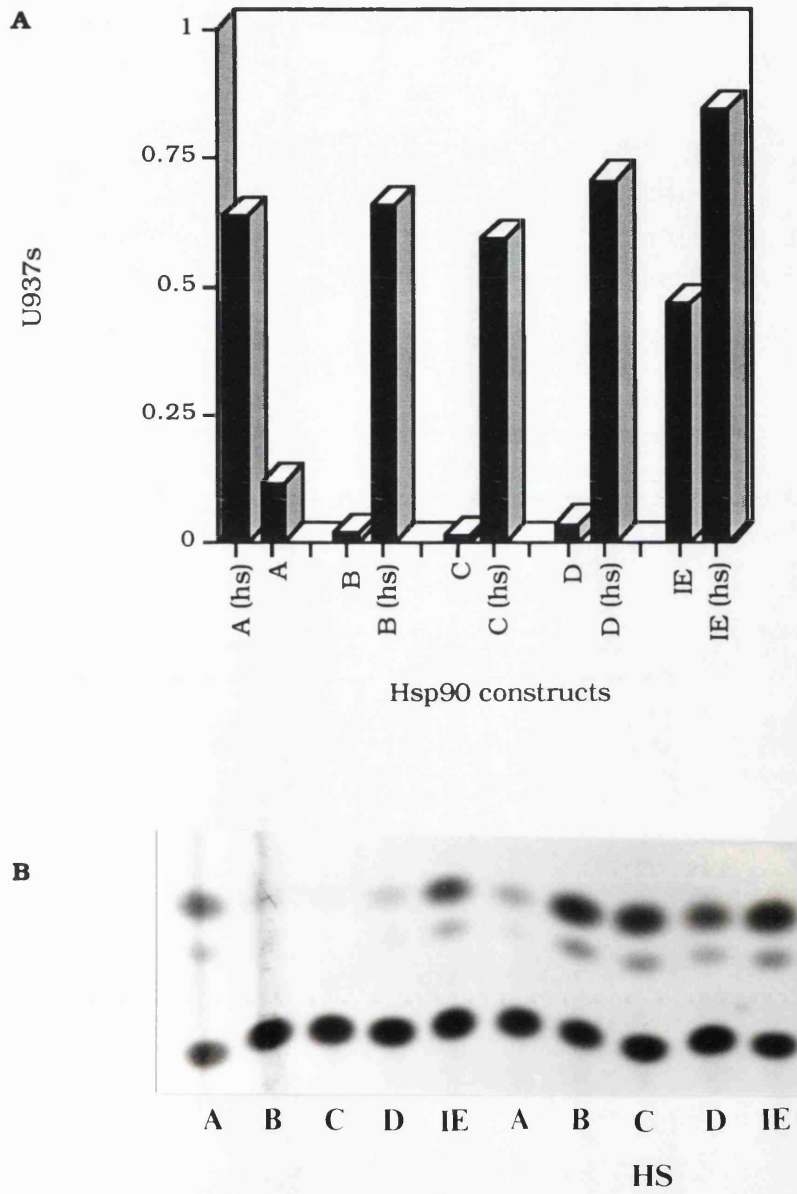
Fig.5.2.1. Expression of all four Hsp90 constructs is dramatically increased under heat shock conditions in the U937 cell line. Note that the photograph does not correspond to the graph, which has been drawn to illustrate the changes in the expression of each construct upon heat shock.

Hsp expression in Jurkat cells is affected by heat shock treatment
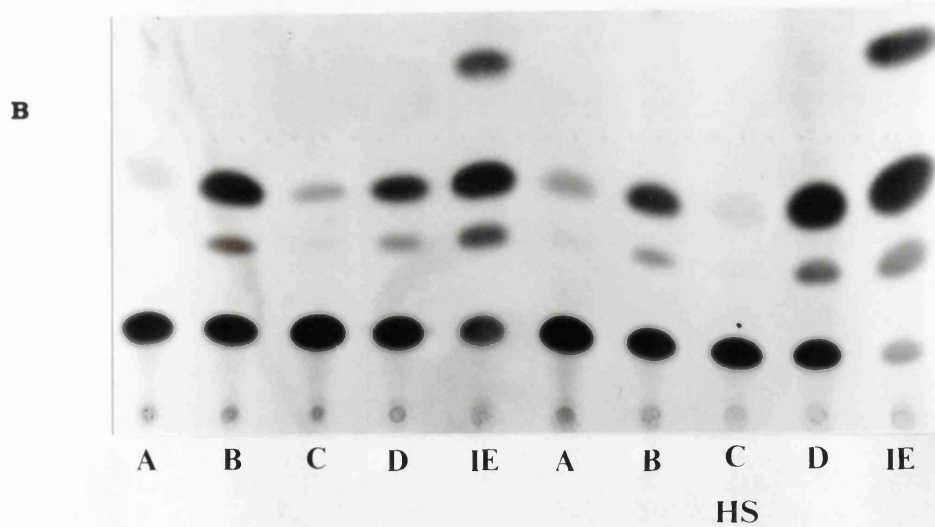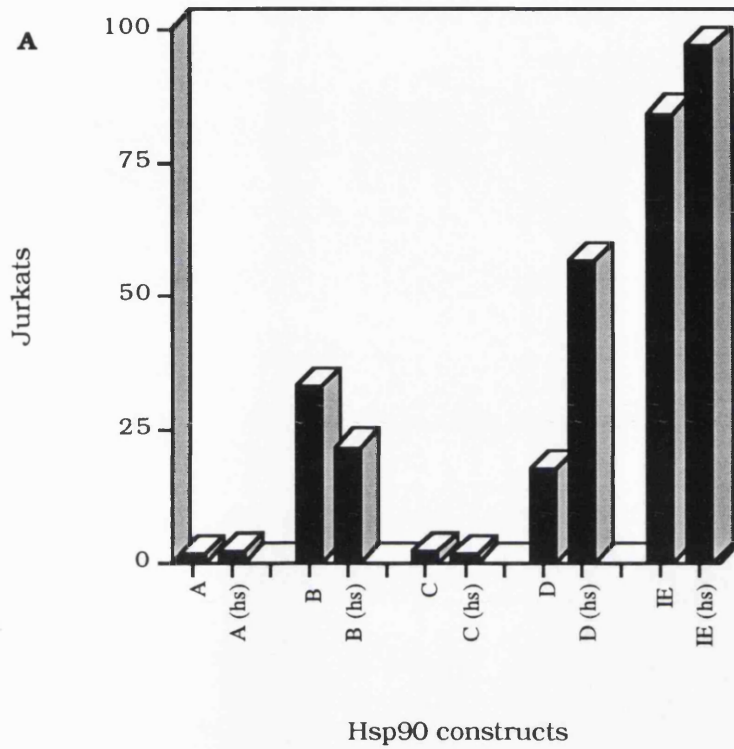
Fig.5.3.1. The B construct in Jurkat cell lines decreases expression on heat shock, despite containing the heat shock elements which mediate increased expression on heat shock treatment.

CHAPTER 6: DISCUSSION

The concept of molecular mimicry, whereby host immune systems respond to proteins from foreign organisms with close homology to self proteins, provided an early hypothesis for the involvement of hsps in the development of autoimmune disease. In this scheme, T cells and antibodies fail to distinguish between host and invading proteins, and autoimmune disease ensues (Oldstone, 1987). Heat shock proteins fulfil many of the requirements to be candidates for molecular mimicry, including ubiquitous expression, high sequence conservation and involvement in the infectious processes of both host and pathogen. In addition, although by no means a common occurrence, there is some evidence for surface expression of hsps or hsp-related proteins (see section 1.2).

The role of heat shock proteins in systemic autoimmune disease remains unresolved, although in autoimmune diseases of unknown aetiology, such as SLE, it is conceivable that a triggering infection might lead to autoimmunity through molecular mimicry. Evidence has been presented that heat shock protein overexpression and antibodies to hsps are relatively common in a number of diseases, but there is so far little evidence to support a hypothesis for a direct role of these proteins in pathogenesis of autoimmunity.

This investigation has focused on whether hsps have any role in the pathogenesis of SLE. Previous studies in our group revealed that a subset of patients overexpressed Hsp90 in their peripheral blood mononuclear cells, and this overexpression correlated with active disease, notably active cardiovascular or central nervous system disease (Norton *et al.*, 1989; Dhillon, 1993). Evidence supporting the observation that Hsp90 is elevated in a subset of patients with SLE has come from other sources (Deguchi *et al.*, 1987). There is thus some abnormal expression or regulation of this protein that appears to be relatively specific for SLE (Dhillon *et al.*, 1993a). Furthermore, autoantibodies to Hsp90

have also been identified in SLE patients (Conroy *et al.*, 1994), a phenomenon which again seems to be relatively disease-specific. While continuing our investigations into human SLE, we can ensure we have access to systems that can be easily manipulated by simultaneously utilising animal models, providing added flexibility in the approach to understanding hsp involvement in this disease. Lupus prone strains of mice provide model systems for experiments that would be difficult or impossible in human patients, as well as providing a testing ground for the development of novel therapeutic strategies.

The MRL/*lpr* mouse is a useful model of SLE, since its range of clinical and laboratory abnormalities provides a close approximation to SLE in humans. This is particularly true for the autoantibody status, as MRL/*lpr* mice generate a range of antibodies specific for SLE that is not paralleled by other models such as NZB/W or BXSB, e.g. anti-Sm antibodies. Furthermore, the disease develops more rapidly compared with the NZB/W model and is more severe in the female mouse, in contrast to the predominantly male BXSB disease.

6.1. Hsp expression in Mrl/*lpr* mice

Increased expression of Hsp90 protein has been demonstrated in the spleens of MRL/*lpr* mice with active disease compared with age and sex-matched MRL/++ and Balb/c mice (chapter 3, section 3.2). This phenomenon appears to be limited to splenic tissue and may be observed in mice as young as three months of age. Since Hsp90 levels are also elevated in a subset of human SLE patients compared with healthy controls a functional role for this protein in the development of disease cannot be ruled out. Elevated Hsp90 levels in SLE patients are associated with active disease in some systems and similarly in MRL/*lpr* mice elevated Hsp90 levels are observed at approximately the time of onset of disease. The mechanisms underlying this increased Hsp90 expression remain obscure however, as does the relationship between Hsp90

overexpression and disease pathogenesis. Heat shock protein levels are influenced by a variety of stressors and hence there are a number of possible explanations for the increased Hsp90 observed in diseased MRL/*lpr* mice, each of which may have some validity with respect to SLE-like disease (Fig.6.1.1).

It is unlikely that elevated Hsp90 levels reflect a simple response to the increased stress of the diseased state, although this may account for the upregulation of Hsp72 observed in a number of tissues of diseased MRL/*lpr* mice, since the increased expression of this protein was observed in a range of tissues (section 3.3). A simple increase in stress fails to explain the tissue specificity of Hsp90 overexpression, however, especially since tissues other than spleen, e.g. the kidneys, are involved in MRL/*lpr* disease. There must therefore be a more specific mechanism underlying the Hsp90 pattern of expression in these mice.

Hsp90 gene transcription levels do not appear to be significantly different in MRL/*lpr* mice compared with healthy MRL/++ and Balb/c mice (section 3.6). Interestingly, this contrasts with elevated Hsp90 levels in SLE patients, which correlate well with enhanced transcription of the Hsp90β gene (Twomey *et al.*, 1993). The lack of enhanced transcription provides further evidence that increased Hsp90 levels are not due to the presence of a known or novel heat shock protein stressor present in the spleen or circulation of MRL/*lpr* mice, since these stresses would be expected to act at the level of transcription through HSFs. The lack of increased transcription does not, however, eliminate the possibility that a different tissue make-up could account for the observed levels of the protein. Since heat shock proteins are known to be expressed at various stages of development, it is feasible that T cell infiltration and proliferation of the dominant double negative T cells could account for increased Hsp90 levels. In support of this, double negative T cells from normal mice are better able to survive heat shock than mature CD4$^+$ or CD8$^+$ cells, a phenomenon which is probably related to relative levels of hsps (Mosser *et al.*,

157

1993). However it remains a matter for speculation, as attempts to generate large enough populations of distinct cell types for analysis by Western blotting proved unsuccessful.

Altered cell composition of the spleen is by no means the only possible source of increased Hsp90. Hsp90 and Hsp72 are differentially expressed over the course of the cell cycle, and this provides another possible mechanism for upregulation of these proteins (Walsh and Li, 1993). However, the unchanged percentage of proliferating cells in MRL/*lpr* mice argues against this being a major contributing factor to hsp overexpression in these animals (Raveche *et al.*, 1982). Another candidate for the source of hsp elevation is an alteration in cytokine expression, IL-2, for example, being a potent inducer of Hsp90 and Hsp70[1] (Granelli-Piperno *et al.*, 1986; Ferris *et al.*, 1988). Although an attractive idea, it seems unlikely that IL-2 plays a major role in the overexpression of Hsp90 in the MRL/*lpr* spleen however, since the predominant cell type in that tissue, the $CD4^-CD8^-$ double negative T cell, lacks functional IL-2 receptors (Tanaka *et al.*, 1993). Other cytokines appear to have similar stimulatory effects on hsp expression in at least some cell types, including IL-1α (D'Souza *et al.*, 1994), IL-1β (Welsh *et al.*, 1991) and TGF-β (Takenaka and Hightower, 1992). Interestingly, TGF-β appears to exert its effects on hsps in a post-transcriptional manner, although the precise mechanism remains unresolved (Takenaka and Hightower, 1992). The TGF-β gene is expressed in $CD4^+$ T cells infiltrating into the kidneys of MRL/*lpr* mice (Mori *et al.*, 1994). However, Hsp90, which is normally induced by TGF-β, was not detected at elevated levels in the kidneys of MRL/*lpr* mouse in this study.

Another candidate for causing the increased Hsp72 levels may be TNF-α, which is a major cytokine produced in various tissues of MRL/*lpr* mice (Mori *et al.*, 1994; D'Souza *et al.*, 1994). Hsp72 overexpression protects against cytotoxicity

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[1] Many of the scientific articles concerning Hsp70 family members do not discriminate between the constitutive Hsp73 or the inducible Hsp72 protein. Every effort has been made to ensure that these proteins are distinguished, but this has not always been possible.

mediated by this cytokine (Jäätela, 1993), and this increased production may be a form of defence mediated by MRL/*lpr* cells against increased expression of this cytokine.

The relationship between hsps and infection is intriguing, as studies have revealed an increased susceptibility to infection in the murine and human disorders (Castro *et al.*, 1993; Lowrance *et al.*, 1994). The increased risk of infection appears to result from spontaneous and improperly regulated production of TGF-β in the murine model (Lowrance *et al.*, 1994) and consequently hsp levels would be expected to be elevated in infected autoimmune mice compared with infected non-autoimmune models. There was little evidence from our colonies of increased infection in MRL/*lpr* mice, however, and it is unlikely that this was a major contributing factor to the elevated Hsp90 levels detected in these animals. Perhaps this may be more relevant in human SLE, as patients have similarly increased susceptibility to infection. Hsps are known to be induced on infection in both host and pathogen, and this induction could result in perpetuation or worsening of the autoimmune disease. Hsps are induced in lymphocytes by mitogens (Haire *et al.*, 1988; Ferris *et al.*, 1988; Hansen *et al.*, 1991), and in MRL/++ mice chronic mitogen stimulation results in early-life glomerulonephritis and development of autoantibodies (Slack *et al.*, 1984).

However, some questions remain unresolved regarding cytokine levels and hsp expression. TGF-β and IL-2 are reputed to have antagonistic effects, yet both induce Hsp70 and Hsp90 expression. In this case, the expression of hsps may simply be a response to altered protein synthesis within the cell, or a natural consequence of their roles as molecular chaperones.

There are several other potential sources of the increased Hsp90 expression. Increased hsp levels may be due to increased mRNA stability, increased translation from the RNA into protein, or possibly decreased degradation of the protein (an increased half-life). There is some evidence in the literature that

stress can result in stability of hsp mRNA (Dellavalle *et al.*, 1994) and this may provide an alternative explanation for the increased expression of Hsp72.

The adenylation state of the mRNA becomes important when considering this possibility, and needs to be addressed before this question can be fully resolved. Adenylated Hsp70 mRNAs appear to be more efficiently translated than deadenylated mRNAs, and deadenylation of the Hsp70 mRNA decreases on heat shock. The rate of degradation of Hsp90 transcripts also appears to be regulated in this manner, correlating with the adenylation state of the mRNA (Dellavalle *et al.*, 1994). With regard to this issue, while a nuclear run-on assay has the advantage of providing information directly from the gene, and thus information about active transcription, a Northern blot would provide a clue as to the relative abundance of the mRNA species, and would indirectly provide information about RNA stability.

The phosphorylation state of Hsp90 also needs to be considered. Given the seemingly fundamental role of antibodies to double-stranded DNA in the pathogenesis of both murine and human lupus, it is interesting to note that dsDNA induces the phosphorylation of Hsp90 on threonine residues (Walker *et al.*, 1985; Lees-Miller and Anderson, 1989b). It is also pertinent to point out that when Hsp90 and Hsp70 bind ATP these molecules undergo a conformational change that results in them being less susceptible to tryptic digestion (Csermely *et al.*, 1993). In addition, the phosphorylation state affects its ability to increase phosphorylation of other proteins (Syzszka *et al.*, 1989).

The significance of the elevated protein remains obscure, although increased Hsp90 expression in the spleens of MRL/*lpr* mice and in peripheral blood mononuclear cells of SLE patients provides indirect evidence for an hypothesis put forward by Srivastava (Srivastava *et al.*, 1994). This model is based on evidence that Hsp90 and Hsp70, as well as the Hsp90-homologous endoplasmic reticulum protein gp96, associate with antigenic peptides derived from cellular proteins, and consists of two main ideas:

(i) Hsps constitute a relay line in which the peptides, after generation in the cytosol by the action of proteases, are transferred from one hsp to another until they are finally accepted by class I MHC molecules in the ER.

(ii) Binding of peptides by hsps constitutes a key step in the priming of cytotoxic T lymphocytes.

In this model, put forward primarily as a result of the authors' interest in immunity and tumourogenesis, hsps are released from virus-infected cells or tumour cells *in vivo* as lysis of cells occurs during infection or by the action of antibodies or non-specific effectors. In the MRL/*lpr* mouse it is easy to envisage the action of antibodies as the major method of release of hsps. These proteins, complexed with antigenic peptides derived from the lysed cells, are taken up by macrophages or other specialised antigen processing cells, possibly via a receptor-mediated event. The hsp-borne peptides are subsequently routed to the endogenous presentation pathway and displayed in the context of the cell's MHC class I, where they are recognised by the precursor cytotoxic T lymphocytes. Since, with regard to autoimmune disease, the immunogenic peptides are self rather than lysed non-self antigenic peptides that would be encountered in infections this would in turn lead to presentation of self peptides by the MHC class I, and hence perpetuation of the disorder.

Indirect evidence that this may be the case is provided by studies of MHC class I-deficient mice. Experimental lupus can be induced in normal mice by immunisation with a human monoclonal antibody to DNA that bears the common idiotype 16/6Id. MHC class I-deficient mice fail to generate any of the classic symptoms of murine lupus, including anti-DNA antibodies, leucopenia, proteinuria or immune complex deposits when immunised in this manner (Mozes *et al.*, 1993). It is also interesting to note that the immunosuppressant Deoxyspergualin binds to both Hsp73 and Hsp90 at concentrations in the range of pharmacologically active doses (Nadeau *et al.*, 1994).

The increased amount of Hsp90 in spleens of MRL/*lpr* mice may also be a consequence of the defect in the Fas antigen, coded for by the *lpr* gene, which is responsible for accelerated illness of these animals. Studies have shown that T lymphocytes from MRL/*lpr* mice rapidly undergo spontaneous apoptosis when purified *ex vivo*, a phenomenon not observed in MRL/++ or C57BL/6-+/+ mice (Van Houten and Budd, 1992). This may be due to a signal for cell death that is unable to be acted upon *in vivo* due to the altered Fas transcripts providing a protective block in the signalling pathway. It is conceivable that Hsp90 acts to chaperone the signalling peptide, which, unable to complete the pathway, remains complexed to the heat shock protein. The heat shock protein may thus be inaccessible to proteases and have a longer half-life within the cell, resulting in the observed increase in Hsp90 levels.

Another functional consequence of elevated Hsp90 levels may be a role in preventing glucocorticoid-mediated apoptosis of "primed" double negative (and other) cells. Heat shock is known to protect cells from glucocorticoid-induced cell death, and this phenomenon is linked with expression of heat shock proteins (Migliorati *et al.*, 1992). It appears that stress-induced elevated Hsp90 levels may influence the hormone binding ability of the steroid receptor (Ali and Vedeckis, 1990). Furthermore, in studies conducted in our laboratory on a number of cell lines Hsp90 levels were shown to increase in response to glucocorticoid (Norton *et al.*, 1989). Glucocorticoid levels may rise as a result of stimulation by IL-1 and IL-6 (Ciavarra and Simeone, 1990), and IL-6 and the IL-6 receptor increase with age in MRL/*lpr* mice (Tang *et al.*, 1991; Kobayashi *et al.*, 1992; Suzuki *et al.*, 1993;). However, glucocorticoids are thought to regulate the production of IL-6 via a inhibitory feedback loop (Waage *et al.*, 1990), and it would appear that some link in this regulatory process has gone awry, as the stimulatory pathway for IL-6, involving IL-1, is apparently normal in MRL/*lpr* mice (Tang *et al.*, 1991). Furthermore, there is no correlation between the use or

Elevated levels of Hsp90 in MRL/*lpr* mice may arise from a number of sources

Stress   Infection

Cytokines

Genetics

Hsp90

increased translation   mRNA stability   Change in cell types

ELEVATED PROTEIN LEVELS
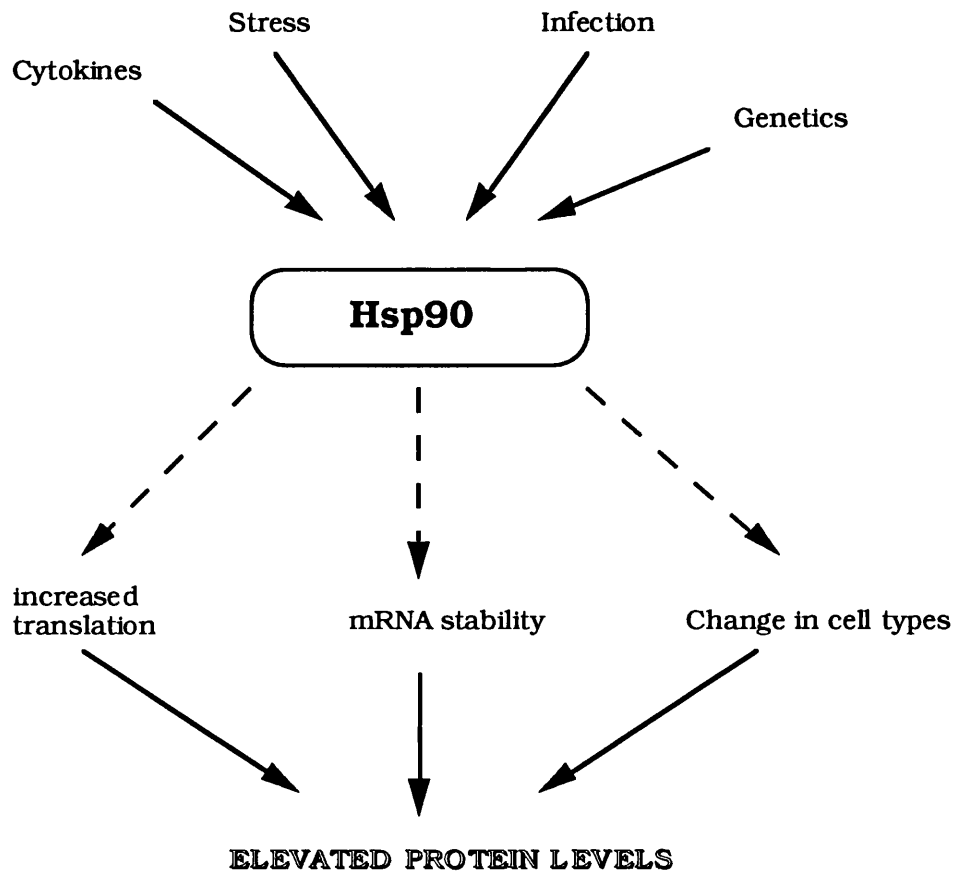
Fig.6.1.1. There are a number of potential sources of the increased Hsp90 levels in the spleens of diseased MRL/*lpr* mice. These act by increasing translation or mRNA stability of the protein, or possibly through causing a change in the tissue's cellular composition. The result is increased expression of Hsp90 compared with the expression of the protein in healthy mice, which in turn may affect the health of the mouse.

extent of steroid therapy and hsp expression in SLE patients (Norton *et al.*, 1989).

The elevated levels of Hsp72 detected in a number of tissues of MRL/*lpr* mice are intriguing. Is there a functional role for this molecule in the MRL/*lpr* disorder? Hsp72 levels appear to be elevated in the mouse kidney, although no cell surface staining of this protein was seen in a study of patients with immunologically based renal disease, including lupus nephritis (Dodd *et al.*, 1993).

The studies described in this thesis have provided little hard evidence that hsps are directly involved in the disease process. It must also be kept in mind that hsps appear to play important roles in the normal "housekeeping" functions of cells. In addition, expression of hsps is dependent on the condition of the cell, and changes over the course of the cell cycle, under conditions of stress (and to different degrees depending on the stress involved) as well as with changes in protein synthesis. Thus, hsp expression is dynamic, fluctuating according to the demands of the cell, and care must be taken before increased levels of these proteins, despite being linked temporally with onset of MRL/*lpr* disease, can be said to be involved directly in the pathogenesis of the disorder.

6.2. Antibodies to hsps in MRL/*lpr* mice

After initial studies showed that the MRL/*lpr* mice from our colonies were comparable to colonies maintained in other laboratories in their production of well-defined autoantibodies like anti-dsDNA and anti-ssDNA, an ELISA protocol for detecting antibodies to the three major heat shock proteins, Hsp90, Hsp70 and Hsp60 was established (sections 4.2, 4.3). Hsps used to coat ELISA plates were not derived from mice, as murine proteins were unavailable commercially when this study was undertaken. The hsps used in these experiments were derived instead from humans, and although there is high species cross-reactivity of autoantibodies (Tan, 1994), there remains an element of uncertainty

regarding the nature of the immunoglobulins detected in this study. Consequently they are referred to in this thesis as antibodies rather than autoantibodies.

Over the course of disease, subsets of MRL/*lpr* and MRL/++ mice generate antibodies to all three heat shock proteins investigated in this thesis, although it is clear that the time courses differ between the two strains. In MRL/*lpr* mice there is an enhanced immune or autoimmune response to Hsp90 that is apparent at or near the time of disease onset as determined by established serological manifestations of MRL/*lpr* disease such as anti-dsDNA antibodies. This response to Hsp90 may appear even earlier, as tracing disease parameters in one mouse revealed an increase in antibodies to Hsp90 from six weeks, while generation of anti-dsDNA antibodies were not evident in this mouse until 10 weeks of age. However, anti-Hsp90 antibodies followed a similar time course to the rise in anti-ssDNA antibodies, which were also evident in this mouse from six weeks. It must also be noted that titres of anti-Hsp90 antibodies[2] generated by this young mouse remained within the mean + 2SD of the average anti-Hsp90 titre of Balb/c until 10 weeks, whereas anti-ssDNA antibodies in the same mouse were well above this range at six weeks.

Antibody titres increased with age and hence disease. Although production of anti-dsDNA and anti-ssDNA antibodies also increases with age there was no correlation between levels of these antibodies and anti-Hsp90 levels. Furthermore, all MRL/*lpr* mice examined over 10 weeks of age had appreciable amounts of anti-ssDNA antibodies, and all had anti-dsDNA antibodies by 12 weeks. In contrast, 60% of the MRL/*lpr* mice over twelve weeks had elevated anti-Hsp90 levels. This finding represents a significant percentage of mice however, comparing favourably with the proportion of MRL/*lpr* mice generating SLE-specific antibody responses such as anti-Sm, with only 37% of males and

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[2] Anti-Hsp90, anti-Hsp70 and anti-Hsp60 antibodies refer to IgG antibodies. References to IgM antibodies are specifically mentioned in the text

10% of females positive at 4-5 months for antibodies to that antigen (Eisenberg *et al.*, 1978)..

It is interesting to note that MRL/++ mice also produce antibodies to Hsp90, although later in development than MRL/*lpr* mice. Elevated levels of anti-Hsp90 antibodies were found in these mice from 30 weeks. In contrast, anti-dsDNA antibodies did not appear in MRL/++ mice until at least 60 weeks of age. Anti-ssDNA antibodies were found at all ages at fairly constant levels, although at higher mean levels than Balb/c mice, until an increase late in life around the time anti-dsDNA antibodies first appeared. It is interesting to again compare these results with published figures for the development of anti-Sm antibodies, which typically appear around 5 months and are found in ~80% of females and ~60% of males by one year (Eisenberg *et al.*, 1978).

The generation of anti-hsp antibodies seems to be a feature of disease onset in both strains of MRL mice, and is apparently linked with the onset of disease. Anti-Hsp70 and anti-Hsp60 levels are correlated in both strains of mice, and it is tempting to speculate that these antibodies are cross-reactive. Cross-reactive antibodies are common in murine lupus, with even the majority of anti-DNA antibodies generally recognising more than one antigen (Banks *et al.*, 1993). However, it should be pointed out that cross-reactivity is not simply a property of polyclonally-derived antibodies, as monoclonal autoantibodies from human hybridomas derived from splenocytes of patients with SLE have been shown to have cross-reactive properties (Ravirajan *et al.*, 1992).

The humoral response to Hsp90 also appears to be different from anti-Hsp70 and anti-Hsp60 production in MRL/*lpr* mice, as anti-Hsp90 antibody levels are not correlated with the levels of the other two antibodies. Anti-Hsp90 IgG antibody levels are correlated with IgM levels in these mice, which may be indicative of an antigen-driven response. In addition, despite there being no correlation between the levels of antibodies to Hsp90 and levels of the other anti-hsp antibodies, there appears to be a subset of mice in which these antibody

levels are related. This may mean that there is a mixed response to Hsp90, with a non-specific subpopulation and a specific antigen-driven component. A complete analysis of the nature of these anti-Hsp90 antibodies would aid in the resolution of this point.

Antibody affinity has not been demonstrated in this study, but given the better results when sera are left overnight to bind to coated heat shock protein on microtitre plates, it is probable that these antibodies are of low affinity. It cannot be presumed that if these antibodies are low affinity they are not involved in the disease process, since low affinity antibodies are often cross-reactive and may be involved in the development of autoimmune disease.

The lack of surface expression of Hsp90 precludes a simple explanation for the generation of anti-Hsp90 antibodies in MRL/*lpr* mice. It may be that the antibody used for these experiments recognises a peptide not presented when these molecules are exposed on the cell surface, or there may be only transient cell surface expression of hsps on the cell surface. The model put forward by Srivastava *et al.* regarding the function of hsps in antigen processing provides two possible explanations for the observed immune response to the hsps:

(i) Upon attack by pathogenic antibodies and cell lysis, hsps may become subject to immune recognition through transient exposure to an extracellular environment. Hsp90 detected on the cell surface of dead cells may thus be the source of antigen necessary to drive an immune response to this protein (see section 3.5). The phenomenon of hsp surface expression on dead cells is not unique, as studies on *post-mortem* tissues have revealed increased staining for hsps with increasing *post-mortem* intervals (Raine, 1994).

(ii) The low titres of anti-hsp antibodies observed in these mice may be a consequence of the fact that these molecules are not highly immunogenic *per se*, but instead are primarily carrier molecules for antigenic peptides. However, since non-specific polyclonal B cell activation is a feature of the MRL/*lpr* disease it is reasonable to assume that there may be a small number of cross-reactive

antibodies which recognise these hsps, possibly as a result of their stated role as carrier molecules or alternatively through conserved epitopes shared with other proteins.

The failure to detect anti-hsp antibodies in a limited study of kidney eluates also provides an argument against a pathogenic role for these antibodies in the development of glomerulonephritis. In fact, autoantibodies need not necessarily have a pathogenic role, and may even have a positive effect. Autoantibodies to Hsp90 have been shown to mediate protection against systemic candidiasis (Matthews *et al.*, 1991), and it is possible that antibodies to hsps may have a similarly beneficial role in the MRL/*lpr* disease.

It is interesting that Hsp60 appears to have little role in the MRL/*lpr* disease. However, a report noting that mycobacterial infection ameliorates MRL/*lpr* disease is intriguing (Castro *et al.*, 1993), since it is generally regarded that mycobacterial Hsp65, or at least a presented peptide of that heat shock protein, stimulates a subset of $\gamma\delta$ T cells. This suggests that recognition of heat shock proteins by the immune system may have a beneficial, as opposed to detrimental effect on the MRL/*lpr* disease.

6.3. Future studies

Many questions remain regarding the role of hsps in the development of MRL/*lpr* disease and human SLE. While it is clear that Hsp90 is expressed abnormally in subpopulations of both murine and human lupus and that antibodies to this protein can also be detected in these disorders, the relationship with the disease process has not been fully elucidated. It has been shown in this study that, temporally, elevated expression of Hsp90 and antibodies to the protein are intimately associated with onset of disease. Whether this is related to pathogenesis or not remains unresolved, and further work needs to be undertaken to determine the exact significance of aberrant hsp expression. The

mechanism by which hsp overexpression occurs needs to be ascertained, as from this study it appears to be unrelated to hsp gene transcription levels in MRL/*lpr* mice, differing from the situation in human SLE. Uncovering the mechanism responsible for the elevated hsp levels would provide further clues as to the source of hsp overexpression.

To aid in the resolution of the mechanism driving Hsp90 elevation it would be useful to determine which of the constituent cell types in the spleen is responsible for the increased expression. Discovering which cells have increased Hsp90 levels would provide an insight into the role of the molecule in the murine disease, and would facilitate a more direct approach to investigating the pathogenic role of Hsp90. The promoter experiments undertaken in this study revealed that a monocytic cell line was capable of inducing heat shock protein constructs to a greater degree than B and T cell lines under heat shock conditions. It would be interesting to pursue this result *in vivo*, although it should be pointed out that hsps may be induced by a number of different stresses other than heat, and that induction of these proteins may arise through different HSFs, each of which is stimulated by a distinct set of stressors (Sistonen *et al.*, 1994; Nakai and Morimoto, 1993).

It would also be of interest to determine which of the Hsp90 proteins, Hsp86 or Hsp84, accounts for the observed increase in protein levels. In human SLE patients it appears to be Hsp90β which is selectively overexpressed, and this protein is the human equivalent of murine Hsp84. In humans Hsp90β appears to be preferentially induced by mitogen stimulation, whereas Hsp90α is upregulated on heat shock (Hansen *et al.*, 1991).

It would be beneficial to establish a transgenic mouse which overexpresses Hsp90 and/or Hsp70. This would be of use in determining whether hsp overexpression is capable of causing disease, although it would seem unlikely, since cells have variable levels of hsps as part of their normal development processes, and hsp overexpression in a stressful context generally has a

protective role. However, it is feasible that chronic hsp overexpression could lead to a breakdown in tolerance, and this is a possibility worth pursuing. In yeast cells overexpressing Hsp90, overabundance of the protein leads to appreciable reduction in growth rate (Cheng et al., 1992).

A pertinent question is whether the antibodies detected in this study are pathogenic. The issue of autoantibody pathogenicity in SLE is a controversial one. It has been suggested that IgG autoantibodies to DNA with an alkaline pI are more pathogenic than those with a lower pI (Theofilopoulos and Dixon, 1985). In addition, one subclass of IgG antibody may be more pathogenic than the other subclasses. IgG_2 antibodies in particular have properties that may contribute to pathogenesis, with the longest half-life, the most efficient consumption of complement and the lowest complement-dependent solubilisation when complexed with antigen of all the subclasses of antibody (Theofilopoulos and Dixon, 1985). This isotype predominates in MRL/lpr mice and in all murine autoimmune strains (Slack et al., 1984).

It would obviously be of interest to determine the exact nature of the anti-hsp antibodies, and to establish whether they fulfil the requirements to be regarded as potentially pathogenic. With regard to this, a set of criteria has been proposed to establish that an autoantibody is pathogenic (Naparstek and Plotz, 1993):

(i) Autoantibody levels and disease activity should correlate.

(ii) The antigen should be found along with a plausible target antigen at the site of tissue damage.

(iii) The autoantibody should be capable of causing the lesions attributed to it in experimental systems.

(iv) A suitable immunisation that leads to the production of similar autoantibodies should lead to a similar disease process.

(v) Removal of the autoantibody should ameliorate the disease process

Clearly much work still needs to be undertaken to determine whether anti-hsp antibodies fulfil these criteria. The first requirement has been demonstrated in

this study, but the remaining points have yet to be established. Purification of the anti-hsp antibodies or generation of antibody-producing hybridomas would facilitate the resolution of some of these questions, and would enable the establishment of an immunisation protocol to directly test the effects of these antibodies in both healthy and diseased mice.

6.4. Conclusion

This study has demonstrated that overexpression of Hsp90, detected in a subset of patients with SLE, is mirrored in the spleen of the autoimmune MRL/*lpr* mouse. In addition, anti-hsp antibodies are found in both MRL/*lpr* and MRL/++ mice with active disease, and both overexpression of the protein and generation of the antibodies appear to be closely associated with the onset and severity of disease. While it is too early to say whether these phenomena have a pathogenic role, this study has provided evidence to suggest that more work needs to be undertaken to elucidate the exact nature of hsp expression in MRL/*lpr* mice and determine whether observed levels are, with regard to disease, cause or effect.

PUBLICATIONS

1.   Gary B. Faulds, David A. Isenberg and David S. Latchman

      The tissue specific elevation of the 90 kDa heat shock protein precedes

      the onset of disease in lupus prone MRL/lpr mice

      J. Rheumatol. 1994: **21** 234-238

2.   S. E. Conroy, G. B. Faulds, W. Williams, D. S. Latchman and D. A.

      Isenberg

      Detection of autoantibodies to the 90kD heat shock protein in SLE and

      other autoimmune diseases

      Br. J. Rheumatol. 1994: **33** (10) 923-927

3.   G. Faulds, S. Conroy, D. Isenberg and D. Latchman

      Increased levels of antibodies to hsps with increasing age in Mrl/Mp-

      lpr/lpr mice

      Br. J. Rheumatol. 1995: *In press*

APPENDIX : BUFFERS AND REACTION MIXES

All chemicals were supplied from BDH unless otherwise stated.

2.2.1 Western blotting

Gel loading buffer
       60mM   Tris-HCl, pH6.8
       10%v/v Glycerol

       5%v/v β-mercaptoethanol
       0.25% Bromophenol blue

Gel running buffer
       0.192M Glycine
       0.025M Tris-HCl, pH8.0
       0.1%   SDS

Blotting buffer
       0.025M Tris-HCl, pH8.0
       0.192M Glycine
       20%   Methanol

Tris-buffered saline (TBS)
       50mM Tris-HCl-pH8.0
       150mM NaCl

2.2.2. Flow cytometry

FITC-labelling buffer
       0.05M Boric acid
       0.2M   NaCl
Adjusted to pH9.2 with NaOH

Dialysis buffer
       0.1M   Tris-Cl, pH7.4
       0.1%   Sodium azide
       0.2M   NaCl
Adjusted to pH7.4 with NaOH

2.2.3 Nuclear run-on assay

10x TKM
       0.5M   Tris-HCl, pH7.5
       0.25M KCl
       0.05M $MgCl_2$

NP40 lysis buffer
       10mM Tris-HCl, pH7.4
       10mM NaCl
       3mM   MgCl2
       0.05% v/v NP40
For cell nuclei, Tris is replaced with 10mM Hepes, pH7.9

Nuclei freezing buffer
       50mM Tris-HCl, pH8.3
       40% v/v Glycerol
       0.1mM EDTA
       5mM $MgCl_2$

L-Broth
For 1 litre of L-broth:
       10g   Bacto-tryptone [Difco]
       5g   Yeast extract [Difco]
       10g   NaCl

Plasmid prep solution 1
    25% w/v   Sucrose
    50mM   Tris, pH8.0
Lysozyme {Sigma} added to a final concentrtion of 1μg/ml fresh before use

Triton buffer
    0.03% v/v Trition X-100
    0.15M   Tris, pH8.0
    0.186M EDTA

Plasmid prep solution 2
    10mM Tris pH8.0
    1mM   EDTA
    1M   NaCl
    20% w/v PEG 6000

Cell resuspension solution
    50mM Tris, pH7.5
    10mM EDTA
    100μg/ml RNAase A

Agarose gels
    1%   Agarose [Life Technologires]
    1X   TBE (see below)
    0.5μg/ml Ethidium Bromide [Sigma]

TBE
    9mM   Tris-HCl, pH8.3
    9mM   Orthoboric acid [BDH]
    2mM   EDTA

TE buffer
    10mM Tris, pH7.5
    1mM   EDTA

Nuclear run-on prehybridisation mix
    0.05M di-Sodium orthophosphate dihydrate
    1 X   Denhardts solution (see below)
    4 x   SSC (see below)
    50% v/v Formamide
    0.2% w/v SDS
    250μg/ml tRNA

100x Denhardts solution
    0.02% w/v ficoll type 400 [Sigma]
    0.02% w/v PVP
    0.02% w/v BSA

Nuclear run on reaction mix
For 2 reactions, the mix was as follows:

128.8μl	H_2O
10μl	1M HEPES
24μl	1M KCl
4μl	1m Dithiothreitol [Boehringer Mannheim]
0.4μl	b-Mercaptoethanol
2μl	500mM Mg Acetate
0.8μl	500mM $MnCl_2$
1.4μl	1M Fructose phosphate
1.6μl	1M Phosphoenolpyruvate
1.6μl	Pyruvate kinase [Sigma]
2μl	Polyoxyethylenesorbitan mono-oleate (Tween 80-[Sigma])
4μl	0.1M 2' Deoxythymidine 3'5' diphosphate sodium [Sigma]
2μl	100mM ATP [Pharmacia, Milton Keynes]
2μl	100mM UTP [Pharmacia]
2μl	100mM GTP [Pharmacia]
2μl	1mM CTP [Pharmacia]
2μl	RNAase inibitor (RNAsin) 1 [Boehringer Mannheim]

Phosphate-buffered saline (PBS)
        104mM NaCl
        1.8mM KCl
        5.4mM di-Sodium orthophosphate dihydrate [BDH]
        1.25mM Potassium dihydrogen orthophosphate [BDH], pH7.0

20x SSC
        150mM NaCl
        15mM   Sodium citrate, pH8.0

2.2.4. ELISAs

Bicarbonate buffer (BIC)
        18.5mM Na$_2$CO$_3$
        7.5mM NaHCO$_3$
pH adjusted to 9.6

2.2.5 Hsp90 Promoter

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

Southern prehybridisation mix
        6x   SSC
        5X   Denhardts
        10% w/v Dextran sulphate [Pharmacia]
        0.1% w/v SDS
        100µg/ml Herring sperm DNA [Sigma]

Oligonucleotide labelling buffer (OLB)
OLB was made from a mixture of solutions -A, B, C and 0
Solution O
        1.25M Tris-HCl, pH8.0
        0.125M MgCl$_2$
Solution A
        1ml   Solution O
        18µl   b-mercaptoethanol
        5µl   0.1M dATP
        5µl   0.1M dTTP [Pharmacia]
        5µl   0.1M dGTP
Solution B
        2M   Hepes, pH6.6
Solution C
        90U/ml Random hexamers in TE [Pharmacia]
OLB was made by mixing A:B:C in a ratio of 100:250:150

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193

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