Differential heat shock protein overexpression and its clinical relevance in systemic lupus erythematosus

Veena B Dhillon, Stuart McCallum, Pamela Norton, Breda M Twomey, Feza Erkeller-Yuksel, Peter Lydyard, David A Isenberg, David S Latchman

Abstract

Objectives—To determine which heat shock proteins (hsps) are overexpressed in systemic lupus erythematosus (SLE), and to examine the relevance of these findings to clinical disease activity.

Methods—Hsp levels in peripheral blood mononuclear cells (PBMC) of patients with SLE and normal controls were measured. Levels were analysed with respect to detailed clinical activity scores. Other hsps were also quantified in 30–50% of these samples.

Results—There was significant increase of the 90 kilodalton heat shock protein (hsp90) in patients with SLE and active neuropsychiatric ($p < 0.005$) and cardio-respiratory ($p < 0.01$) disease. There was also significant increase of the inducible 72 kilodalton member (hsp72), but not the constitutive 73 kilodalton member (hsp73) of the hsp70 family, and no increase of the 60 kilodalton hsp (hsp60) was seen in patients compared with controls. There was no association of hsp72 with disease activity, and no correlation between hsp90 and hsp72 levels was seen in individual patients.

Conclusion—There may a specific role for hsp90 in distinct, clinically active subsets of patients with SLE.

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Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease primarily affecting women of childbearing years. The aetiology probably involves a mosaic of genetic, hormonal, dietary, and complement deficiency factors.¹ Its prevalence varies from 1:250 among black women in the West Indies and the United States, to 1:4300 in white subjects (estimated) in New Zealand. The clinical manifestations of SLE are protean, and there is notable mortality and morbidity—treatment with major immunosuppressive drugs often being required for many years. The diversity of clinical features is matched by an apparent diversity of autoantibodies detectable in the serum of patients. A broad range of T cell abnormalities has also been demonstrated.²

Hsps were initially identified on the basis of their increased synthesis following exposure to raised temperatures.^{3–4} They have since been

shown to be synthesised at increased levels after exposure to a variety of stressful stimuli. Identical proteins (isoforms) are also present constitutively, having a variety of critical functions which can be seen to be readily adapted to protect the cell from damage.⁵

In normal cells, hsp90 is therefore intracytoplasmic, involved with holding intracellular steroid (glucocorticoid, oestrogen, progesterone) receptors in conformations that prevent the receptor from binding to nuclear DNA until hormone/receptor interaction has occurred.^{6–7} Members of the 70 kilodalton hsp family appear to prevent inappropriate interactions of proteins which have not reached their final stage of assembly into multiprotein complexes;⁴ they may also be involved in translocation of proteins within the cell and in antigen presentation.⁸ Hsp60 aids the transport of proteins into mitochondria by catalysing their unfolding.⁵

Hsps thus function as common rapid response 'chaperones' for intramolecular and cell membrane events, as well as controlling the transfer of cytoplasmic signals to the nucleus.⁹ They are vital to the survival of the cell and this idea is supported by their remarkable phylogenetic conservation.^{4–10}

Hsps have been shown to be immunodominant antigens during bacterial and mycobacterial infections.^{11–12} Indeed, primitive human T cell repertoires include clones that are directed against hsps, and these may play a beneficial part as a first line of defence against such infections.¹³ In addition, low levels of autoantibodies to hsp60 have been found in essentially all human serum samples.¹⁴

Hsps may be at the interface between tolerance and autoimmune disease, if the potentially autoreactive T and B cells described above are triggered to react with endogenous hsps. This could be brought about by increased expression and perhaps cellular relocation of endogenous hsps, not only as a disease-initiating event but also as a result of the stresses associated with continuing autoimmune tissue damage.

We have made the preliminary observation that hsp90 is raised in peripheral blood mononuclear cells (PBMC) in 15–20% of patients with active SLE, compared with normal controls and patients with rheumatoid arthritis.¹⁵ This increase was shown to be independent of the presence of fever or of steroid treatment. Significant overexpression

University College and Middlesex School of Medicine,
University College, London,
United Kingdom
Division of Molecular Pathology
V B Dhillon
S McCallum
P Norton
B M Twomey
D S Latchman

Department of Rheumatology Research
D A Isenberg

Department of Immunology
F Erkeller-Yuksel
P Lydyard

Correspondence to:
Professor David Isenberg,
Bloomsbury Rheumatology Unit,
Arthur Stanley House,
Tottenham Street,
London W1P 9PG,
United Kingdom.

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of hsp90 in active SLE has also been documented by others in studies of small numbers of patients.^{16,17}

In view of the heterogeneity of SLE among patients and within individual patients over time we examined this overexpression further. The objective was to determine whether the raised levels of hsp90 seen in SLE were specific for this protein or whether the other hsps were also increased in parallel. In addition, we wanted to know what the relation was between increased hsps and the disease process.

We therefore examined the expression of other hsps—hsp60, the highly inducible 72 kilodalton (hsp72) and the constitutive 73 kilodalton (hsp73) members of the hsp70 family⁴—in patients with SLE compared with normal controls. Hsp90 was quantified in PBMC from patients over a four year period. Hsps60, 72, and 73 were quantified in a number of these samples, and compared with results from normal controls.

Patients, materials and methods

MATERIALS

Mouse monoclonal antibody (MoAb) AC88 (gift from D Toft), developed after isolation of hsp90 from *Achyla ambisexualis*, reacts with human hsp90.¹⁸ AC88 also reacts with a number of 30–40 kilodalton proteins, shown to be heterogeneous nuclear ribonucleoproteins,¹⁹ and so western blotting was employed for hsp90 quantification rather than the simpler enzyme linked immunosorbent assay (ELISA).

Mouse MoAb 4B9/89 (gift from M Sharif and G Rook) reacts with human hsp60 (M Sharif, PhD thesis, University of London, 1991).

Mouse MoAbs to human hsp72 and hsp73 were obtained from StressGen Biotechnologies Corporation, Sidney, British Columbia, Canada.

PATIENTS AND CONTROLS

One hundred and two patients (97 women, five men; mean age 38.9, range 17–70) who fulfilled the American Rheumatism Association's 1982 revised criteria for the classification of SLE, were studied.²⁰ A total of 222 samples of venous blood were taken, during morning outpatient attendances at the Bloomsbury Rheumatology Unit.

Fifty nine normal control subjects (47 women, 12 men; mean age 30.3, range 16–65)—healthy volunteers consisting of hospital and laboratory staff—were studied. A total of 102 blood samples were taken, all in the morning.

Hsp90 was quantified in all of the above samples. Hsp60 was quantified in 43 and 33, hsp72 in 64 and 58, and hsp73 in 72 and 54 of the SLE and control samples respectively.

ISOLATION OF PBMC

Fresh heparinised venous blood (20 ml) was diluted 1:1 with Roswell Park Memorial Institute Medium (RPMI) (Sigma, Dorset,

United Kingdom), and PBMC isolated by centrifugation on Ficoll-Paque (Pharmacia, Milton Keynes, United Kingdom). PBMC were resuspended in 1 ml RPMI and counted.

SEPARATION OF MONONUCLEAR CELL POPULATIONS

Some experiments necessitated the separation of PBMC into T, macrophage/monocyte and non-T cell populations. This was achieved by a combination of adherence to plastic plates at 37°C (monocyte/macrophage fraction), and T cell rosetting with neuraminidase-treated sheep red blood cells (Tissue Culture Services, Milton Keynes), followed by centrifugation through Ficoll-Paque (Pharmacia), and incubation of non-T and T cell fractions with red cell lysis buffer (155 mM ammonium chloride, 10 mM ammonium bicarbonate, 0.1 mM EDTA). Protein samples were prepared from cell fractions thus harvested as described for PBMC.

PREPARATION OF PROTEIN SAMPLES

PBMC were pelleted in a microfuge at 6000 rpm for one minute, and resuspended in sample buffer containing 2.3% sodium dodecyl sulphate (SDS), 0.0625 M Tris, 10% glycerol, 5% β-mercaptoethanol, and bromophenol blue at a concentration of 10⁶ cells to 50 µl sample buffer. Samples were sonicated for 10 s at 2 A, boiled for two minutes and stored at –20°C.

PROTEIN ELECTROPHORESIS AND IMMUNOBLOTTING

The method used was that of Norton *et al*,¹⁵ with minor modifications. Protein samples were subjected to vertical, one dimensional SDS-polyacrylamide gel electrophoresis (PAGE). The lower gel consisted of 7% acrylamide (in 1.5 M TRIS, 0.4% SDS, pH 8.8), and the stacking gel consisted of 5% acrylamide (in 0.5 M TRIS, 0.4% SDS, pH 6.8). Samples of 50 µl (10⁶ cells) per track were loaded and electrophoresed in duplicate on two halves of the same gel, in running buffer (0.192 M glycine, 0.025 M TRIS, 0.1% SDS, pH 8.3), for 1.5 h at 30 mA through the stacking gel, and 2.5 h at 45 mA through the lower gel.

Each gel was cut in half—one half was stained and destained with Coomassie blue, and the other half underwent western blotting in circulating blotting buffer (0.192 M glycine, 0.025 M TRIS, 20% methanol, pH 8) for 15 h at 210 mA.

Western blots to be incubated with AC88 were blocked with 3% bovine serum albumin (BSA), TRIS buffered saline (TBS), and 0.05% Tween-20 for 1 h at room temperature, washed thrice in 0.3% BSA, TBS, 0.05% Tween-20 (buffer A), and once in TBS for five minutes each. Blots for incubation with the other MoAbs were blocked with 2% BSA, TBS, 0.2% Tween-20 (buffer B) for 1 h at room temperature, washed thrice in this solution, and once in TBS and Tween-20 for five minutes each.

Blots were incubated with the MoAb of choice (AC88 at 12.5 µg/ml in buffer A, 4B9/89 at 0.125 µg/ml in buffer B, StressGen MoAbs at 0.5 µg/ml in buffer B) for 2 h at room temperature, and washed as above.

Hsp90 blots were incubated with ¹²⁵I labelled antimouse F(ab')₂ fragments raised in sheep (Amersham International, Buckinghamshire, United Kingdom), at 25 000 cpm/ml in buffer A for 2.5 h at room temperature. They were washed twice in buffer A for 10 minutes each, and finally in TBS for 10 minutes. The blots were dried and autoradiographed using X-OMAT AR film (Kodak, Hemel Hempstead, United Kingdom), with multiple exposures of 3–20 days at –70°C.

Hsp60, 72, and 73 blots were incubated with antimouse immunoglobulins (raised in rabbit) conjugated to horseradish peroxidase (Dakopatts, Glostrup, Denmark) at a 1 in 300 dilution in buffer B for 2 h at room temperature, and washed as described above. Blots were developed using an enhanced chemiluminescence kit (Amersham), with multiple autoradiograph exposures of 5–30 s at room temperature.

HSP MEASUREMENT

Figure 1 shows an autoradiograph of a representative western blot demonstrating hsp90 bands and the corresponding stained/destained gel. Hsp bands on the autoradiographs and actin bands on the corresponding stained/destained gels were scanned using the VD620 densitometer (Biorad, Hemel Hempstead, United Kingdom) within the linear range.

Levels of hsp were calculated as a ratio of the positive control standard (Jurkat T cell line), which constitutively overexpresses hsps, and which was run on all gels, equalising for inter-gel and interblot variation. It was shown that there was a linear relation between hsp band density and the amount of Jurkat protein loaded ($R^2=0.95$).

Doubling dilution experiments also verified that both the number of cells and the total amount of protein loaded per track were directly proportional to the density of the actin band ($R^2=0.98$ and 0.99 respectively). Actin (as the most abundant cellular protein and one with a constant level of expression) was therefore used to equalise hsp values of all samples for small differences in protein loading.

Thus the formula used to calculate the hsp90 level of a sample was:

$$\frac{\text{hsp90 band of sample (OD} \times \text{mm)}}{\text{actin band of sample (OD} \times \text{mm)}} \bigg/ \frac{\text{hsp90 band of Jurkat (OD} \times \text{mm)}}{\text{actin band of Jurkat (OD} \times \text{mm)}} \times E$$

where E is the equalisation factor obtained by calculating the hsp90 level of the current generation of Jurkat (stored in aliquots at –20°C) against the first generation. Hsp levels are therefore expressed in arbitrary units, and are independent of the number of cells or the amount of protein loaded onto a track of the gel.

The maximum expected error (mean +2SD) for each hsp90 value was 0.04.²¹ This was calculated from 44 paired observations obtained under different experimental conditions, such as using different amounts of Jurkat standard, different generations of Jurkat standard, different exposures of autoradiograph, or different runs of the same sample on different occasions. The consistency of the Jurkat standard was demonstrated by the fact that different amounts and different generations of Jurkat produced the smallest errors (maximum expected error 0.008 and 0.009 respectively).

ASSESSMENT OF CLINICAL DISEASE ACTIVITY

As a routine part of their management, detailed clinical data were recorded and laboratory parameters were requested at the time of sampling of patients' blood for this study. It was therefore possible to assign disease activity scores retrospectively to 95% of the SLE samples. Each sample was scored by one of us (DAI) who was blind to the hsp results.

Figure 1 Measurement of hsps by western blotting. Representative autoradiograph of immunoblot, with its corresponding stained/destained gel. Autoradiograph shows hsp90 bands. Gel shows actin bands. Tracks 1–8 are; 1—Jurkat standard; 2—normal control sample; 3 to 8—SLE samples.

Results were initially analysed using the overall University College Hospital/Middlesex scoring system.²² SLE is a multisystem disease, however, with extremely variable expression both within individual patients over a period of time, as well as within populations of patients. We therefore also used the British Isles Lupus Assessment Group's (BILAG) system for analysing disease activity.²³ This has been validated internationally as well as against other computerised methods of scoring SLE activity.^{23 24 25} BILAG defines activity within each of eight disease categories or organ systems: scores A or B denote active disease; C and D denote barely noticeable and no or never any disease respectively. (E has been added subsequently to distinguish no previous disease).

We compared active (A or B) with inactive (C or D) disease in each of the eight disease categories—for example, active mucocutaneous SLE was compared with inactive mucocutaneous SLE. We were thus able to examine the relation between hsp levels and each of eight individual disease categories in which SLE could be active or inactive in a patient at the time of sampling.

STATISTICAL ANALYSIS
As both SLE and normal control raw data were skewed, significance testing was carried out using two tailed Student's unpaired *t* test on log₁₀ normalised data. Correlations were performed using Spearman's rank correlation method.

Results
The table shows the mean (2 SEM) and 95% confidence limits for each hsp in patients with

Mean hsp levels in SLE and control PBMC				
	Hsp90	Hsp72	Hsp73	Hsp60
SLE	0.13	17.69	0.39	0.06
Controls	0.07	7.64	0.30	0.07
p Value*	<0.001	<0.001	>0.7	>0.3

Hsp levels are expressed in arbitrary units (see methods); *Level of significance of difference between means of SLE and control groups; 95% Confidence limits (2SEM) of mean SLE hsp90, hsp72, hsp73 and hsp60 were 0.11 to 0.15, 14.17 to 21.21, 0.26 to 0.53 and 0.04 to 0.09 respectively; 95% confidence limits of mean normal control hsp90, hsp72, hsp73 and hsp60 were 0.06 to 0.09, 5.58 to 9.7, 0.18 to 0.42 and 0.03 to 0.11 respectively.

SLE and controls. Hsp90 and hsp72 were significantly raised in patients (*p*<0.001), whereas hsp60 and hsp73 were not. There was no significant correlation between raised hsp90 and hsp72 in individual SLE samples, nor indeed between levels of any of the hsps (data not shown). This suggests that overexpression of one hsp does not automatically mean that there is overexpression of other hsps in an individual patient sample.

Figure 2 shows that there was considerable variation in hsp90 levels in macrophage/monocyte, T and B cell populations fractionated from PBMC of different SLE samples, even in those patients with raised levels of PBMC. This suggests that simple alteration in the proportion of one cell type with naturally high hsp90 in PBMC from patients with SLE cannot account for our observation of raised hsp90 in these patients. In contrast, there was a fairly consistent pattern of hsp90 expression in the cell types fractionated from PBMC of normal controls, regardless of the hsp90 level in their PBMC—the T cell fractions expressing hsp90 to the highest levels.

Hsp90 was significantly raised in patients with severely active disease compared with patients with inactive or mildly active disease

Figure 2 Heat shock protein (hsp90) levels in cell populations isolated from peripheral blood mononuclear cells (PBMCs). Hsp90 levels in T, B, and macrophage/monocyte cell populations fractionated from PBMCs of five representative patients with SLE and four normal controls.

($p < 0.025$ and $p < 0.05$ respectively), using the UCH/Middlesex scoring system (data not shown). This confirms our original findings.¹⁵ There was no significant increase of hsp72 in active disease scored by this method (data not shown). There was no correlation of either hsp90 or hsp72 with ESR or anti-double stranded DNA (anti-dsDNA) antibody titre in individual SLE samples. Patients taking oral prednisolone, antimalarial drugs, or azathioprine did not have significantly different levels of hsp90 or hsp72 levels compared with patients not taking these drugs (data not shown).

Figure 3 shows that patients with active neuropsychiatric SLE according to the BILAG scoring system had significantly raised hsp90 compared with patients with inactive neuropsychiatric SLE ($p < 0.005$). Similarly, patients with active cardiorespiratory disease had significantly raised hsp90 compared with patients with inactive cardiorespiratory SLE ($p < 0.01$). There was no significant difference in hsp90 levels in patients with active compared with inactive SLE in any of the other six BILAG disease categories. Neither hsp72, nor hsp73, nor hsp60 were significantly higher in the active category than in the inactive disease categories (data not shown). Indeed, hsp73 appeared to be higher in the inactive disease categories, significantly so in inactive neuropsychiatric SLE ($p < 0.02$) and inactive renal SLE ($p < 0.025$), compared with active disease in these categories. Thus hsp90 alone is significantly raised in certain clinically active subsets of patients with SLE.

Serial studies in individual patients showed that in 21 patients neuropsychiatric or cardiorespiratory SLE, or both, shifted from active to inactive or vice versa. In 12 of these patients (57%), hsp90 levels clearly went from high

($> \text{mean controls} + 2\text{SD} = 0.2$) to low when either or both of these categories became inactive (seven patients), or became high when either or both of these categories became active (five patients).

Discussion

There are compelling arguments to support the hypothesis that hsps are involved in autoimmune phenomena. As cytoplasmic molecules with a physiological role in protein folding and unfolding, endogenous eukaryotic hsps are obvious candidate molecules for a key role in determining the way in which cells interact with the immune system. There is evidence to suggest that members of the hsp70 family may have a critical role in antigen processing.⁸ Expressed genes encoding the hsp70 family have been shown to be located within the major histocompatibility complex (MHC),²⁶ and expressed genes encoding the hsp90 family have been located near to the MHC class I region²⁷ and the immunoglobulin heavy chain locus.²⁸ In addition, mitogen activation of T cells produces enhanced synthesis of hsps90 and 73.²⁹

Immune recognition of exogenous prokaryotic hsps may lead to the breakdown of tolerance to endogenous hsps, especially if the latter are being overexpressed. This may occur as a result of bacterial infection, or of an intrinsic aberration in the genetic regulation of hsp expression.

A crucial event linking overexpression of particular endogenous hsps to the breakdown of tolerance would be the expression of hsps on the cell surface. We now have evidence that surface expression of hsp90 does indeed occur on PBMC of patients with active SLE, compared with both normal controls and with

Figure 3 Hsp90 levels and activity in BILAG disease categories. Mean hsp90 levels (with bars showing 95% confidence limits) for active (A or B) and barely noticeable or inactive (C or D) disease in each of the eight BILAG disease categories. Muco/cut=mucocutaneous; NP=neuropsychiatric; Mus/skel=musculoskeletal; CR=cardiorespiratory; Vasc=vasculitis; Haem=haematological.

patients with inactive SLE.³⁰ In that study there also appeared to be a subgroup of patients in whom overexpression of hsp90 correlated to a degree with surface expression of hsp90. The lack of hsp60 elevation in SLE demonstrated in the current study is matched by lack of surface expression of hsp60 as shown by FACS analysis. In addition, the heterogeneity of hsp90 overexpression by different cell populations in any one patient shown in fig 2³¹ is matched by similar evidence from these surface expression studies.

A consequence of overexpression or surface expression of hsp90 might be the development of anti-hsp90 autoantibodies. This is currently the object of further investigation in our laboratories, with studies being performed upon serum from the same blood samples in which hsps were quantified in this study.

A criticism of efforts to test the hypothesis that hsp overexpression may play a part in autoimmunity, is that one might expect all hsps to be overexpressed in concert merely as a result of the continuing disease process—a 'stressful' situation. The evidence presented here suggests that this is not the case.

We have shown that hsp90 is overexpressed in 32% of patients with SLE (if mean of controls + 2SD = 0.2 is taken as the upper limit of 'normal'). This is a significant number of patients in a disease as heterogeneous as SLE, and is similar to the number of patients with lupus who, for example, have antibodies to Sm and RNP.³²

There is a marked, significant increase of hsp90 in patients with active, compared with inactive neuropsychiatric and cardiorespiratory SLE. Although hsp72 is overexpressed in SLE, there is no correlation between hsp90 and hsp72 in individual patients, and there is no relation between hsp72 and disease activity. In addition, we have shown that hsp60 and hsp73 are not raised in SLE. We have also reported that the small hsp ubiquitin is not overexpressed in SLE.³³ The lack of correlation of hsp90 levels with ESR or anti-dsDNA titre implies that the hsp90 increase is not simply a marker of overall active disease. These data argue for a specific role for hsp90 in particular, clinically active subsets of SLE.

With regard to the specificity of these findings to SLE, we have considerable data regarding differential hsp over- and under-expression in a variety of other diseases (Dhillon *et al*, unpublished observations and ref 34.) The pattern of hsp90 and 72 over-expression, but no elevation of hsps60 or 73, appears to be specific to SLE. We did not find significant hsp90 overexpression in patients with RA,¹⁵ nor did we find significant hsp90 overexpression in Behçet's syndrome or recurrent oral ulceration—two conditions in the pathogenesis of which herpes simplex virus type I may play a part.³⁴

The findings presented here, of differential hsp overexpression in SLE, and of hsp90 elevation in certain clinically active subsets of SLE, together with the finding that all cell types in SLE appear to be capable of hsp90 overexpression (fig 2 and ³¹), suggest that there

may be an underlying genetic alteration in regulation of hsp90 expression, particularly in SLE. Indeed, raised levels of hsp90 correlate with raised transcription rates of this protein in patients with SLE (Twomey *et al*, unpublished data).

In the current study we have also shown that hsp90 shifts in accord with neuropsychiatric or cardiorespiratory disease activity, or both, in 12/21 (57%) of patients with SLE. Although the relation of these disease activities taken individually, to hsp90 levels, is difficult to interpret, these figures are comparable with those from previous attempts to correlate laboratory parameters with disease activity in SLE. For example, using a combination of circulating immune complex levels, dsDNA binding, lymphocyte counts, and C3 levels, statistical analysis by discriminant function showed that only 44% of cases of SLE could be correctly classified into their clinical disease activity categories.³⁵

To our knowledge there are no reports which examine in detail the comparative expression of hsps, and their relation with disease activity, in autoimmune rheumatic diseases such as SLE. Clearly it is now of importance to understand the processes which produce the specific increase in hsp90 in clinically active subsets of SLE, and their consequences for cellular function and the autoimmune response, in order to elucidate the role of this increase in the aetiology and pathogenesis of SLE.

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