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Identification of human heat shock protein 60 (Hsp60) and anti-Hsp60 antibodies in the peripheral circulation of normal individuals

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Although primarily regarded as being intracellular, this study has identified the presence of heat shock protein 60 (Hsp60) in the peripheral circulation of normal individuals. The median Hsp60 concentration was approximately 3.5-fold higher in females than in males and significantly higher levels of anti-human Hsp60 antibodies were also detected in females. There were no differences in the levels of antibodies to mycobacterial Hsp60 between males and females, nor did antibody levels correlate with Hsp60 concentrations. Hsp60 was not released from mitogenically stimulated peripheral blood mononuclear cells. The potential physiological roles for circulating Hsp60 are unknown. Given the emerging evidence that inappropriate reactivity to heat shock proteins is involved in autoimmune disease and that T cells responsive to self Hsp60 appear to be protective, these findings suggest that circulating Hsp60 may be involved in the regulation of tolerance to self and immunity to bacterial forms of these widely expressed and structurally conserved proteins.

INTRODUCTION

Heat shock or stress proteins belong to several families based on molecular weights and are among the most phylogenetically conserved proteins. The stress response is a universal event and is induced by a range of conditions including increased temperature, exposure to oxidative stress, ultraviolet irradiation and viral infection (Garry et al. 1983; Subjek and Shyy 1986; Donati et al. 1990; Suzuki and Watanabe 1992; Omar and Pappolla 1993; Welch 1993). A precise function for these molecules remains unclear, however they appear to be involved in repair processes and to have a number of cellular functions such as the assembly and folding of oligomeric proteins and their intracellular translocation (Hightower 1991).

Heat shock proteins appear to provide a link between infection and autoimmunity. The similarity between microbial and mammalian forms of the molecules may lead to an anti-human response either through recognition of conserved epitopes or via cross-reactivity termed molecular mimicry (Lamb et al. 1989). Reactivity to heat shock proteins has been implicated in a range of autoimmune diseases including arthritis (Res et al. 1988; Gaston et al. 1990; de Graeff-Meeder et al. 1991), multiple sclerosis (Wucherpfennig et al. 1992; Georgopoulos and McFarland 1993; Stinissen et al. 1995) and diabetes (Elias et al. 1990; Child et al. 1993; Tun et al. 1994). A heat shock protein-mediated component to vascular disease has also been suggested and increased levels of anti-mycobacterial Hsp60 antibodies are found in individuals with vasculitis, arteriosclerosis and borderline hypertension (Frostegård et al. 1997; Gruber et al. 1996). Elevated anti-mycobacterial Hsp60 antibody titres also appear to be associated with the severity of disease (Xu et al. 1993).

Although heat shock proteins are primarily regarded as being localized intracellularly, their release from cultured rat embryo cells and human islet cells has been reported (Hightower and Guidon 1989; Child et al. 1995). Albeit uncertain, it has been suggested that heat shock protein release may be an altruistic response to protect

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neighbouring cells that are less capable of producing these proteins (Hightower and Guidon 1989).

This study reports the development of an enzyme immunoassay for Hsp60 and the identification of Hsp60 immunoreactivity in the peripheral circulation of healthy individuals. This observation is intriguing and the physiological basis for circulating Hsp60 is currently unknown. Immunization with recombinant heat shock proteins can protect against disease in experimental models of arthritis (Van den Broek et al. 1989; Billingham et al. 1990; Thompson et al. 1990; Van Eden et al. 1995) and reactivity to self-Hsp60 appears to confer protection (Van Roon et al. 1996). The findings of this study raise the possibility that circulating Hsp60 may be a component of a complex regulatory mechanism that is involved in the maintenance of peripheral tolerance to these highly conserved and widely expressed molecules.

MATERIALS AND METHODS

Serum samples

Circulating Hsp60 concentrations and anti-heat shock protein antibody levels were determined in serum samples (*n*=60) obtained from overtly healthy individuals donating blood to the Trent Centre of the National Blood Transfusion Service, Sheffield. Serum was kept at 4°C for no longer than 48 h prior to subsequent storage in aliquots at −80°C. Individuals taking antibiotics and certain other drugs were excluded, as were pregnant women and women planning a pregnancy. Menstruating women were not excluded from donating blood if they were not anaemic. However, the possibility that menstruation and sloughing of the endometrium may have contributed to elevated levels of serum Hsp60 observed in females was unlikely given that there were no differences in serum Hsp60 concentrations between pre- and post-menopausal women (data not shown). The age distributions of the males and females included in the study were equivalent (Table 1).

Enzyme immunoassay for Hsp60

Ninety-six well microtiter plates (Nunc-Immuno Plate MaxiSorp) were coated with a murine monoclonal antibody to human Hsp60 (clone LK.1; StressGen Biotechnologies Corporation, Canada) in carbonate buffer (pH 9.5) overnight at 4°C (100 µl/well; 2 µg/ml). This monoclonal antibody recognizes an epitope between 383 and 447 of the human Hsp60 molecule and shows no reactivity with equivalent bacterial molecules (Boog et al. 1992). Coated plates were washed with phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBS/T) using an automatic plate washer and non-specific binding sites were blocked by incubation with 150 µl of PBS/T containing 1% bovine serum albumin (Sigma Chemical Co., UK) for 1 h at room temperature. After washing, 100 µl of the reference preparation (recombinant human Hsp60; 0–2500 ng/ml; StressGen) or samples were added and the plates incubated for 1 h at room temperature. Plates were washed and bound Hsp60 was detected by adding 100 µl rabbit polyclonal anti-Hsp60 antibody (StressGen; 1/1000) in PBS/T containing 2% mouse serum (Sigma). Plates were washed after 1 h and

Table 1 Hsp60 concentrations and anti-human Hsp60 (hHsp60) and anti-mycobacterial Hsp60 (mHsp60) antibody levels in males and females

Sex (age range)	*n*	Hsp60 (ng/ml)	Anti-hHsp60 (Aunits/ml)	Anti-mHsp60 (Aunits/ml)
Males (23–63)	32			
Mean ± SD		1725 ± 2032	133 ± 75	388 ± 231
Median		663	117	315
(range)		(95–7573)	(18–430)	(122–1130)
Females (21–55)	28			
Mean		3849 ± 3534[a]	240 ± 185[b]	474 ± 328[ns]
Median		2481	174	408
(range)		(138–14679)	(58–750)	(131–1375)

For antibody determinations, all samples were pre-screened and the two having the highest level of antibodies to human Hsp60 and mycobacterial Hsp60 were identified. These samples were assigned a concentration of 1000 arbitrary units/ml and were used to generate standard dose-response curves for the respective assays. Data were transformed ($\log_{10}$) prior to comparative statistical analyses.

a) Significant difference between males and females (*P*=0.0011; Student's *t*-test for unpaired data);

b) Significant difference between males and females (*P*=0.0038; Student's *t*-test for unpaired data).

n.s.–not significant.

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incubated with 100 µl of alkaline phosphatase-conjugated anti-rabbit immunoglobulin murine monoclonal antibody (1/10,000 in PBS/T; Sigma) for 30 min. Binding of conjugated antibody was detected by adding 200 µl of *p*-nitro phenyl phosphate (*p*-NPP) substrate (Sigma). The resultant absorbance was determined at 405 nm using a Titertek Multiscan MCC/340 plate reader.

Enzyme immunoassay for anti-human and anti-mycobacterial Hsp60 antibody levels

Anti-heat shock protein antibody levels were determined essentially as described previously (Danieli et al. 1992). Briefly, microtiter plates were coated with recombinant human Hsp60 (StressGen) or *M.bovis* 65kDa antigen (mHsp60; 100 µl/well; 2 µg/ml in carbonate buffer). Recombinant mycobacterial Hsp60 was obtained from UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases. Coated plates were blocked and washed as described above. Test serum (typically 1/100) was added and the plates incubated for 1 h at room temperature. Bound heat shock protein antibodies were detected by adding 100 µl of alkaline phosphatase-conjugated polyclonal goat anti-human lgA/G/M (1/10,000) in PBS/T. Plates were washed and after 1 h, 200 µl of *p*-NPP substrate (Sigma) was added and the absorbance determined at 405 nm.

All sera were pre-screened and a sample having a high level of anti-human Hsp60 or anti-mycobacterial Hsp60 was identified. These samples were assigned a concentration of 1000 arbitrary units/ml (Aunits/ml) and were used to generate standard dose-response curves, from which antibody levels in the remaining sera were determined using ASSAYZAP data analysis software (BIOSOFT, Cambridge, UK).

Effect of mitogenic stimulation on Hsp60 release

Given that cultured rat embryo and human islet cells have been shown to release heat shock proteins (Hightower and Guidon 1989; Child et al. 1995), we determined whether Hsp60 is released from in vitro stimulated peripheral blood mononuclear cells. Mononuclear cells were isolated from heparinized whole blood by density gradient centrifugation and suspended at 10^6 viable cells/ml in RPMI 1640 growth medium supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin, 2 mM L-glutamine (Life Technologies Ltd, UK) and 10% fetal bovine serum. Isolated cells were stimulated with the T cell mitogen Concanavalin A (Con A; 5 µg/ml) in 96 well culture plates (5 replicates per time point) and at the times indicated, 100 µl of culture supernatant were removed and pooled appropriately. Samples were centrifuged and stored at −20°C until assay for Hsp60 concentration.

The activation/proliferative response of the stimulated cells was determined in the same cultures using a colorimetric assay (Mosmann 1983). Briefly, 10 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) solution (5 mg/ml in PBS) was added to each well. After 3 h at 37°C, 100 µl of acidified isopropanol (0.04 M HCI) containing 0.3% w/v SDS (Sigma) was added to dissolve the formed crystals. The absorbance was determined at 570 nm and used as a measure of cell activation/proliferation. Cell viability was assessed in parallel cultures by trypan blue dye exclusion.

Statistical analysis

Hsp60 concentrations and heat shock protein antibody levels demonstrated a lognormal distribution, as a consequence of which data were transformed (log10) prior to comparative and correlative statistical analyses. Comparisons between Hsp60 concentrations and anti-heat shock protein antibody levels in males and females were performed using the Student *t*-test for unpaired data. Correlations between Hsp60 concentration, heat shock protein antibody levels and age were performed using the non-parametric Spearman rank test. Statistical comparisons were performed using SPSS and MultiStat statistical analysis software.

RESULTS

Evaluation of the Hsp60 assay

A representative standard curve for the Hsp60 assay is shown in Figure 1. The background of the assay was low and typically less than 0.01 absorbance unit (Fig. 1). The intra-assay precision was determined by assaying 12 replicate dilutions (0–2500 ng/ml) of the recombinant human Hsp60 standard. Duplicate dilutions acted as the standard dose-response curve and the concentrations of the remaining 10 sets were determined by reference to the standard curve. The variability was expressed as the coefficient of variation (CV) and was approximately 5% at concentrations greater than 100 ng/ml (Fig. 1). The sensitivity of the assay was defined as the concentration that gave an absorbance twice that of the zero standard control and was 27.4 ng/ml. The inter-assay variability was determined by assaying samples on separate occasions and was typically less than 10% over the working range of the assay (data not shown). The assay did not detect mycobacterial Hsp60.

Establishment of an Hsp60 normal range

Serum Hsp60 concentrations displayed a lognormal distribution and data were transformed (log10) prior to

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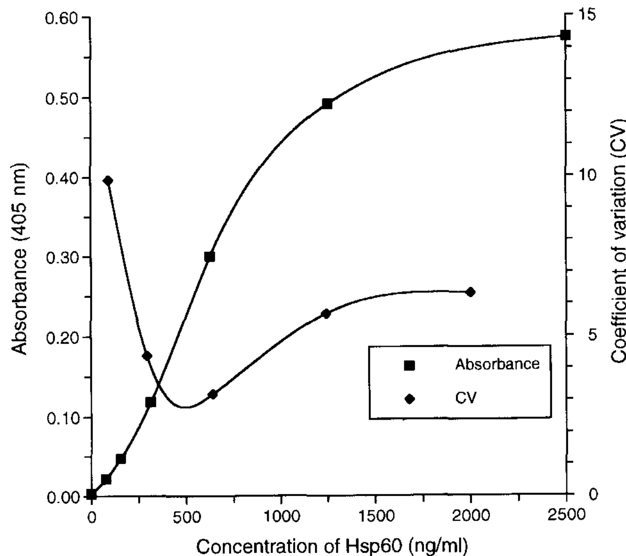

Fig. 1 Representative standard dose-response curve and intra-assay precision profile for the Hsp60 enzyme immunoassay. Precision was determined by assaying 10 replicate dilutions of recombinant human Hsp60 standard. The variability of the results at the individual concentrations is expressed as the coefficient of variation (CV). The sensitivity of the assay (27.4 ng/ml) was defined as the concentration that gave an absorbance twice that of the zero standard.

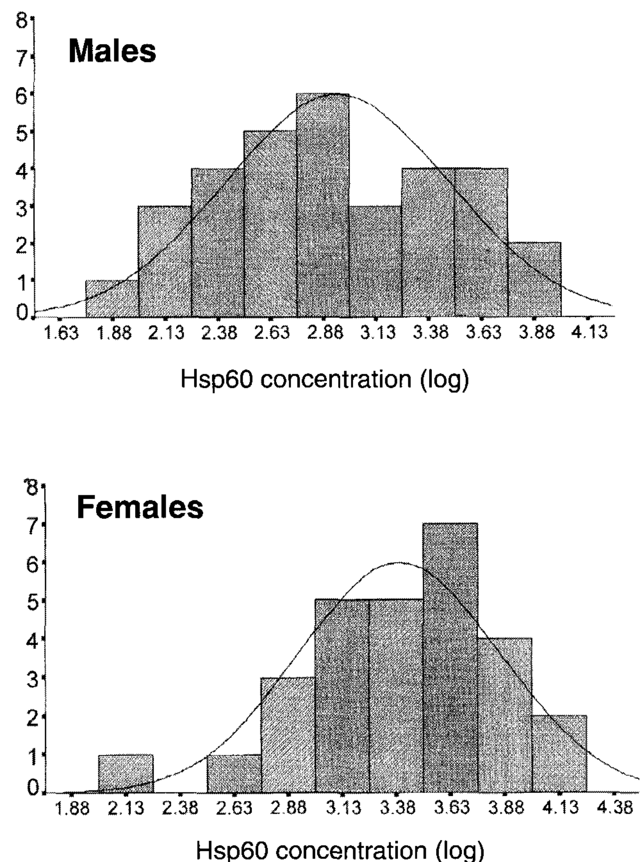

Fig. 2 Lognormal distribution of Hsp60 levels in the peripheral circulation of males (upper; *n*=32) and females (lower; *n*=28).

analysis (Fig. 2). Hsp60 was detectable in all serum samples studied and median concentrations were significantly higher (~3.5-fold) in females (Table 1). As a consequence of this finding, correlation of Hsp60 concentrations with age and levels of heat shock protein antibody was performed for males and females separately. A trend towards decreasing serum Hsp60 concentrations with increasing age was observed in both males and females, however this failed to achieve statistical significance (data not shown).

Anti-heat shock protein antibody levels

Anti-human Hsp60 and anti-mycobacterial Hsp60 antibodies were detected in all samples (Table 1). As observed for serum Hsp60, antibody levels demonstrated lognormal distributions and anti-human Hsp60 antibody levels were significantly greater in females (Table 1). There were no differences in levels of anti-mycobacterial Hsp60 antibodies between males and females (Table 1).

Hsp60 release from in vitro activated peripheral blood mononuclear cells

Given the evidence that heat shock protein synthesis can be induced following T cell activation with mitogens or cytokines (Lanks 1986; Lindquist 1986; Craig 1993), it may be that Hsp60 is released following in vitro mitogenic stimulation of peripheral blood mononuclear cells. However, despite a potent activation response that peaked around day 5, Hsp60 was not detectable in cell culture supernatants at any of the time points investigated (data not shown). These data indicate that Hsp60 is not released as a consequence of immune cell activation, however they do not exclude the possibility that limited secretion does occur, but at levels undetectable by the assay or that Hsp60 may be released following cell death by apoptosis or necrosis.

DISCUSSION

Although typically regarded as being intracellular, heat shock protein release from cultured rat embryo and human islet cells has been demonstrated (Hightower and Guidon 1989; Child et al. 1995). This study reports the presence of Hsp60 and anti-human and anti-mycobacterial Hsp60 antibodies in the peripheral circulation of normal individuals. Given the sequence homology of the

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human and mycobacterial Hsp60 it is likely that the polyclonal anti-heat shock protein antibody response is cross-reactive and anti-human Hsp60 antibodies recognize mycobacterial protein and vice versa. This may explain the observed lack of correlation between protein and antibody levels.

Albeit speculative at this stage, a physiological basis for circulating heat shock protein may be the maintenance of peripheral tolerance. It is now clear that thymic deletion does not eliminate autoreactive T cells from the peripheral circulation (Hämmerling et al. 1993). Hsp60-specific T cells are present in normal individuals (Munk et al. 1989; Ramage et al. 1997) and endogenous heat shock protein induces T cell reactivity in patients with juvenile chronic arthritis (de Graeff-Meeder et al. 1991). These findings suggest that effective regulation of potentially deleterious autoreactive T cell populations is required.

The precise mechanism by which peripheral or extrathymic tolerance is achieved and maintained is a subject of debate. It may result from clonal inactivation (anergy), clonal deletion or by a balance of cells secreting pro-inflammatory (Type 1) and regulatory (Type 2) cytokines. It may also be that potentially self-reactive T cell populations escaping deletion in the thymus fulfil important regulatory roles in the maintenance of peripheral tolerance (Cohen 1992).

It is clear that tolerance can be broken and autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), the animal corollary to multiple sclerosis, and arthritis can be induced. Adjuvant arthritis, which is induced using heat-killed *M. tuberculosis*, is T cell driven and can also be initiated by T cell clones specific for mycobacterial Hsp60 (Van Eden et al. 1988). The potential influence of heat shock protein reactivity on the pathogenesis of clinical autoimmune disease has been suggested by the identification of mycobacterial Hsp60-specific T cells in the joints of patients with rheumatoid arthritis (Res et al. 1988; Gaston et al. 1990).

In contrast to their potentiating effects, heat shock proteins can also protect against disease – as demonstrated by the modulation of experimental arthritis by mycobacterial Hsp60 and Hsp70 (Van den Broek et al. 1989; Billingham et al. 1990; Thompson et al. 1990; Kingston et al. 1996). It is interesting to note that protection by Hsp60 appears to be elicited by autoreactive T cells recognizing specific sequences of self stress proteins (Anderton et al. 1995, Anderton and Van Eden 1996).

An insight into the possible mechanisms by which self heat shock proteins may modulate autoimmune disease has come from the work of de Graeff-Maeder et al. (1995). This group has demonstrated that in patients with juvenile chronic arthritis, in whom the disease follows a remitting rather than progressive course, the presence of circulating T cells responsive to human Hsp60 appears to be beneficial. The human (self) Hsp60 responsive T cells were of the regulatory Type 2 phenotype, whereas reactivity to the 65 kDa mycobacterial antigen Hsp65 reactivity displayed the inflammatory Type 1 phenotype and appeared to correlate with disease severity (Van Roon et al. 1996).

Given the observations that heat shock protein reactive T cells are present in the peripheral circulation and that self-heat shock protein reactivity appears to down-regulate autoimmune responses to heat shock proteins, it is tempting to speculate that circulating Hsp60 are involved in the regulation of potentially harmful self-heat shock protein reactivity and the maintenance of tolerance. This proposition is supported by the observation that human, but not bacterial Hsp60 can stimulate suppressive responses by T cells from the synovial fluid of patients with rheumatoid arthritis (Van Roon et al. 1997). This would suggest that any tolerogenic or suppressive effects of circulating heat shock proteins are targeted towards pro-inflammatory Type 1 T cells via a stimulation of regulatory Type 2 T cells.

Clearly, further studies are required to more definitively characterize the Hsp60 molecule, its interaction/association with antibody and the role of anti-heat shock protein antibody in potentiation or prevention of disease pathogenesis. These studies are currently underway, as are investigations into circulating heat shock proteins in individuals with established vascular and autoimmune disease.

In summary, the implications of these findings have yet to be fully evaluated. However, given the sequence homology between mammalian and bacterial forms of these molecules and the ubiquitous nature of their expression, it would seem reasonable to propose that complex regulatory mechanisms capable of maintaining tolerance to self and immunity to bacterial heat shock proteins would be required. The findings of this study may lead to a renewed insight into the physiological role of heat shock proteins and reactivity to heat shock proteins in health and disease.

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