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Heat shock proteins form part of a danger signal cascade in response to lipopolysaccharide and GroEL

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Summary

An increasing number of cell types, including peripheral blood mononuclear cells (PBMCs), have been demonstrated to release heat shock proteins (Hsps). In this paper we investigate further the hypothesis that Hsps are danger signals. PBMCs and Jurkat cells released Hsp70 (0·22 and 0·7 ng/10^6 cells, respectively) into medium over 24 h at 37°C. Release of Hsp70 was stimulated 10-fold by GroEL ($P < 0·001$) and more than threefold by lipopolysaccharide (LPS) ($P < 0·001$). Although Hsp60 could be detected in the medium of cells cultured at 37°C for 24 h, the low rates of release were due probably to cell damage. Significant release of Hsp60 was observed when Jurkat cells were exposed to GroEL (2·88 ng/10^6 cells) or LPS (1·40 ng/10^6 cells). The data are consistent with the hypothesis that Hsp70 and Hsp60 are part of a danger signalling cascade in response to bacterial infection.

Keywords: danger signals, GroEL, Hsp60 release, Hsp70 release, LPS, lymphocytes, peripheral blood mononuclear cells.

Introduction

Responses to bacterial infection involve recognition of foreign antigens and a signalling cascade alerting, or priming, the immune system. Recently heat shock proteins (Hsps) have been implicated as members of a danger signalling cascade [1–3]. This paper examines whether Hsp70 and Hsp60 could form part of the response to the bacterial antigens LPS and GroEL.

Bacterial-derived LPS and Hsps are stimulators of the innate immune system [4–7] and have been demonstrated to initiate activation and cytokine release from monocytes, macrophages and dendritic cells [8–14]. Innate immune responses to bacterial LPS and Hsps have been demonstrated to occur through CD14/TLR2/TLR4 receptors on antigen-presenting cells [15–18]. Responses to mammalian Hsp60 and Hsp70 have been reported to occur through both CD14-dependent and CD14-independent pathways [7,19–21].

Hsps are normally intracellular proteins that have functions involved in protein folding and maintenance of protein integrity under both normal and stress conditions, yet if they are to act as signals in response to infection they would need to be present in the extracellular environment. A number of bacterial Hsps (such as the chaperonin 60 protein of *Escherichia coli*, GroEL) have been found in extracellular locations and implicated in immune reactions to infection [22–25]. Human Hsps have also been shown to be present on cell surfaces [26–28], in serum [29–32] and to be released from a variety of cell types [33–38]. We have reported recently the release of Hsp70 from lymphocytes [36].

In order for Hsps to act as danger signals there is a requirement to demonstrate that their release can be up-regulated in response to stresses that would occur *in vivo*, such as bacterial infection; for Hsp60 and/or Hsp70 to have a role as a danger signal we would predict that bacterial antigens, such as LPS and GroEL, would stimulate their release. This paper provides evidence for this stimulation in both PBMCs and cultured Jurkat cells.

Materials and methods

The Biological Sciences Ethics Committee (University of Chester) approved the study. Whole blood was taken from healthy volunteers and informed written consent was obtained from all subjects.

Isolation of cells from whole blood.

Whole blood was diluted 1 : 1 in phosphate buffered saline (PBS; 0·14 M, pH 7·4) and subjected to density centrifugation using Histopaque® (Sigma 1077–1, Poole, UK). Peripheral blood mononuclear cell (PBMC) pellets were resuspended

(~2×10^7 cells/ml) in RPMI medium (Sigma, R-8758) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% antibiotic/anti-mycotic solution (Sigma, A-5955).

Jurkat cell culture

Jurkat E6·1 human T cell leukaemia cells were cultured in RPMI-1640 medium (Sigma, R-8758) supplemented with 10% fetal calf serum (Fetalclone I; Perbio Science, Cramlington, UK) 2 mM L-glutamine (Sigma, G-7513) and 1% antibiotic/anti-mycotic solution (Sigma, A-5955). Cells were grown at 37°C, 5% CO_2. Experiments were performed on cells that had achieved a density of ~ 4×10^5 cells/ml (mid-log expansion), and contained < 1% of dead cells as determined by Trypan blue staining.

Giant cell tumour cell (GCT) culture

GCT stromal cells were cultured in α-Minimum Essential Medium (MEM) (Sigma, M-8042) supplemented with 10% fetal calf serum (Fetalclone I; Perbio) 2 mM L-glutamine (Sigma, G-7513), and 1% antibiotic/anti-mycotic solution (Sigma, A-5955). In preparation for experiments 1 ml cells were seeded into 12-well plates at a density of ~2×10^5 cells/ml, and were incubated at 37°C in 5% CO_2 for 3 days or until confluent. Medium was removed from the confluent cells and replaced with 1 ml of supplemented α-MEM medium.

Cell treatments

Prior to treatment PBMCs and Jurkat cells were resuspended in 10 ml supplemented RPMI and washed by centrifugation (500 ***g***, 3 min at room temperature). Medium was removed from the pellet and the cells were resuspended in supplemented RPMI to give a density of ~ 3×10^6 cells/ml (Jurkat) and ~ 2×10^7 cells/ml (PBMCs). Cell suspensions were transferred into 12-well plates (1 ml/well) and t_0 (time zero) samples were harvested immediately. Cell treatments consisting of Hsp60 (10 μg/ml) (NSP-540; Stressgen, Victoria, Canada), Hsp70 (10 μg/ml) (Stressgen, NSP-555), GroEL (10 μg/ml) (Stressgen, SPP-610-G) and LPS (10 μg/ml) (Sigma, L-4516), diluted in the appropriate medium, were added to the remaining wells in triplicate and the cells were incubated at 37°C, 5% CO_2 for 24 h.

Measurement of released Hsp60 and Hsp70

Following treatments Jurkat and PBMCs were harvested by centrifugation (500 ***g***, 3 min at room temperature). Cell lysates were prepared from the pellet as below for the measurement of intracellular Hsp60, Hsp70 and lactate dehydrogenase (LDH). The culture medium was removed from each cell pellet and transferred to clean centrifuge tubes. The media obtained were centrifuged (500 ***g***, 3 min at room temperature), decanted and transferred to clean tubes. This was repeated twice, and the media were checked to ensure that no cells remained in the samples. Medium from GCT cells was removed from each well by pipette, and cells were resuspended in 1 ml PBS. All samples were stored at –80°C prior to measurement. Hsp60 and Hsp70 were quantified in medium and cell extracts using Hsp60 and Hsp70 enzyme-linked immunosorbent assay (ELISA) kits (Stressgen Biotechnologies, EKS-600 and EKS-700).

Western blotting

Cell lysates and media were suspended in 1 ml of extraction buffer [0·1% Triton X-100, 100 mM KCl, 8 mM $MgCl_2$, 150 mM NaCl, 20 mM Tris-HCl pH 7·4 and 1 mM phenylmethylsulphonyl fluoride (PMSF)].

The cellular and media samples (10 μl per sample) and a control sample (5 μl), recombinant human Hsp60 control (Stressgen, ESP-540E) or recombinant human Hsp70 (Stressgen, ESP-555E), were diluted 1 : 1 in sodium dodecyl sulphate (SDS) sample buffer, boiled for 3 min, then separated on 8% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. The blots were blocked for 1 h in 1% b ovine serum albumin (BSA) in Tris-buffered saline (2·85 g Tris, 29·24 g NaCl, in 1 dm^3, pH 7·5) and then probed with the relevant primary antibody, murine anti-Hsp60 monoclonal antibody (Stressgen, SPA-806) or murine anti-Hsp70 monoclonal antibody (Stressgen, SPA-810) at a concentration of 1 μg/ml for 1 h at room temperature. The primary antibody was detected using anti-mouse IgG peroxidase (Sigma, A-5278) at a concentration of 1 : 1000 for 1 h at room temperature. The blot was developed using metal enhanced 3, 3′-diaminobenzidine (DAB) substrate (Pierce, 34065, Illinois, IL, USA).

Lactate dehydrogenase (LDH) assay

A reaction mixture was prepared consisting of: 2·6 ml glycine buffer, 0·6 M, pH 9·2 (Sigma, 826–3), 3 mg sodium lactate (Sigma, L-7022), 10 mg nicotinamide adenine dinucleotide (NAD) (Sigma, 260–110) and 3·4 ml distilled water. Media samples (50 μl/well) and cell extracts (50 μl/well) were added to a 96-well microplate alongside LDH standards (Sigma, 826–6). The reaction mixture was added to the plate (100 μl/well) and the plate read at 340 nm at 2-min intervals up to 30 min.

Cell counting and viability staining

Total cell counts and cell viability counts were carried out on all samples before and after treatments using a haemocytometer. Counts to determine cell viability were performed by diluting the suspended cell sample 1 : 1 with Trypan blue (Sigma, T-8154); cells that took up the blue stain were counted as non-viable.

Statistical analysis

All data are presented as the means ± standard errors of three separate experiments. Difference between sample means was

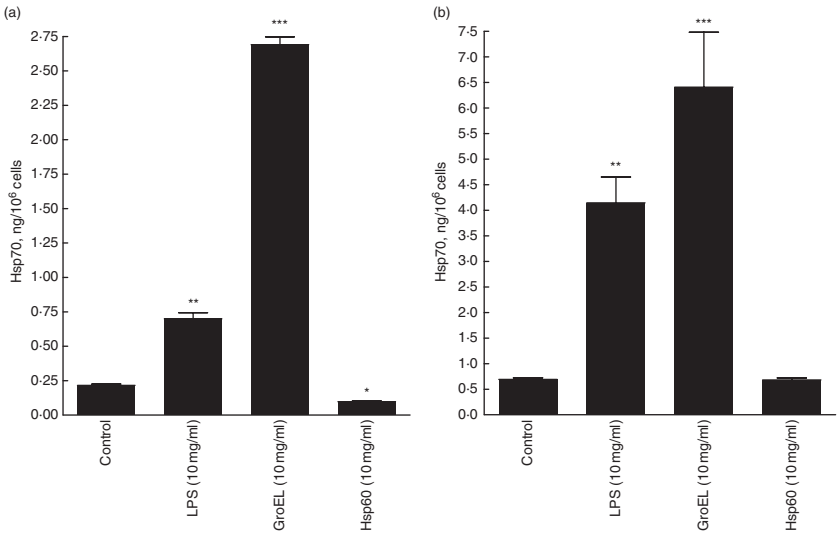

Fig. 1. Heat shock protein (Hsp)70 release into culture media from peripheral blood mononuclear cells (a) and Jurkat cells (b) following incubation with lipopolysaccharide (LPS) (10 μg/ml), GroEL (10 μg/ml) and Hsp60 (10 μg/ml) for 24 h. Data presented as mean ± s.e.m., $n = 3$. *Indicates significant difference from 37°C control; *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

evaluated through use of Student's *t*-test or Dunnett's post hoc multiple comparison test.

Results

PBMCs incubated at 37°C for 24 h released Hsp70 (Fig. 1a). LPS caused a significant increase ($P < 0{\cdot}001$) in Hsp70 release (Fig. 1a). Incubation with GroEL also resulted in a significant increase ($P < 0{\cdot}001$) in Hsp70 release (> 10-fold) (Fig. 1A). Incubation with Hsp60 for 24 h caused a small reduction ($P < 0{\cdot}05$) in Hsp70 release from PBMCs (Fig. 1a). Jurkat cells incubated at 37°C for 24 h also released Hsp70 (Fig. 1b). Jurkat cells showed a similar pattern of Hsp70 release to PBMCs in response to Hsp60, GroEL and LPS treatments (Fig. 1b): LPS caused Hsp70 release to increase ($P < 0{\cdot}01$); GroEL caused a 10-fold increase ($P < 0{\cdot}001$); Hsp60 had no significant effect on Hsp70 release.

PBMCs incubated at 37°C for 24 h released low amounts of Hsp60 (Fig. 2a). Following 24 h incubation of PBMCs with LPS, Hsp60 release was significantly increased ($P < 0{\cdot}01$) (Fig. 2a). Incubation with GroEL or Hsp70 caused significant increases ($P < 0{\cdot}001$) in Hsp60 release from PBMCs (Fig. 2a). Jurkat cells incubated at 37°C for 24 h released Hsp60 (Fig. 2b). Incubation of Jurkat cells with LPS resulted in a rate of Hsp60 release which was not significantly different from the control (Fig. 2b). However, incubation with GroEL caused a significant increase ($P < 0{\cdot}001$) in Hsp60 (Fig. 2b). Treatment with Hsp70 resulted in a >10-fold increase ($P < 0{\cdot}001$) in Hsp60 release (Fig. 2b).

Western blots confirmed that the Hsp60 and Hsp70 release responses observed from PBMCs (Fig. 3) and Jurkat (data not shown) were due to the release of complete 60 kDa and 70 kDa Hsps. The intracellular enzyme LDH was used as a control for damage related protein release. In control cells

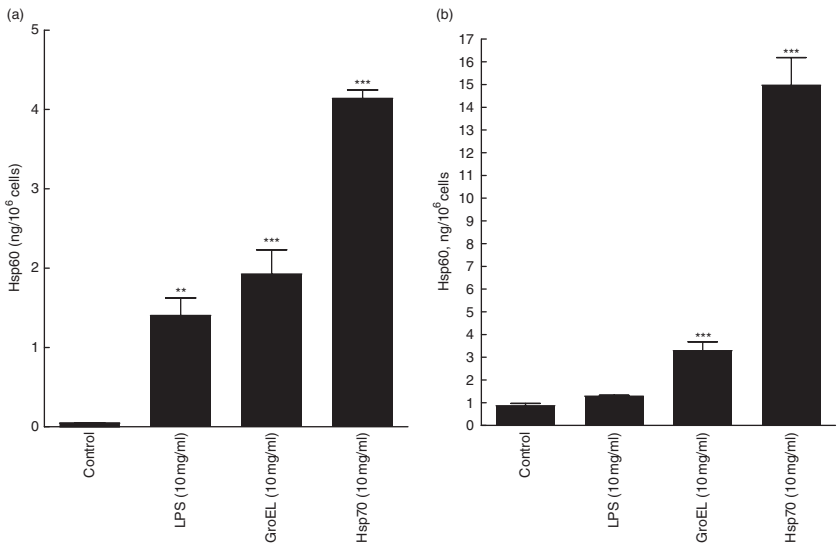

Fig. 2. Heat shock protein (Hsp)60 release into culture media from peripheral blood mononuclear cells (a) and Jurkat cells (b) following incubation with lipopolysaccharide (LPS) (10 μg/ml), GroEL (10 μg/ml) and Hsp70 (10 μg/ml) for 24 h. Data presented as mean ± s.e.m., $n = 3$. *Indicates significant difference from 37°C control; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$).

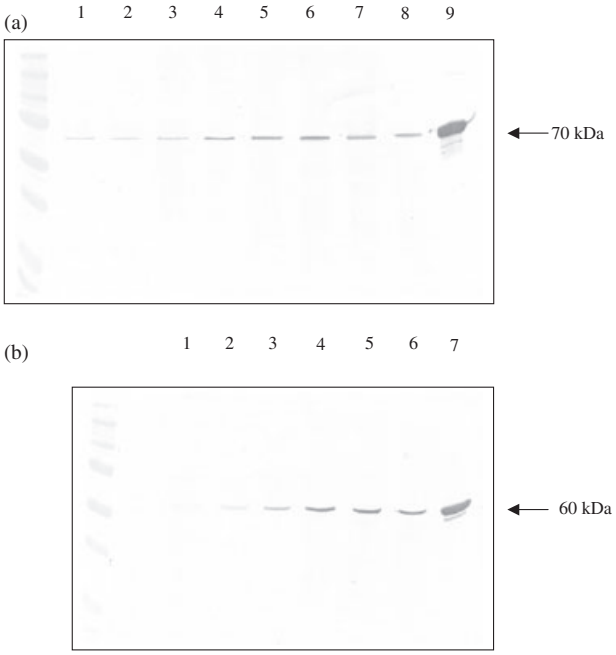

Fig. 3. Western blots showing the presence of heat shock protein (Hsp)60 and Hsp70 in peripheral blood mononuclear cells media and cell extracts following 24 h treatment with Hsp60, Hsp70, GroEL and lipopolysaccharide (LPS). (a) Hsp70 in media: lane 1, control; lane 2, Hsp60 (10 μg/ml); lane 3, LPS (10 μg/ml); lane 4, GroEL (10 μg/ml). Hsp70 in cells: lane 5, control; lane 6, Hsp60 (10 μg/ml); lane 7, LPS (10 μg/ml); lane 8, GroEL (10 μg/ml); lane 9, Hsp70 standard. (b) Hsp60 in media: lane 1, control; lane 2, LPS (10 μg/ml); lane 3, Hsp70 (10 μg/ml). Hsp60 in cells: lane 4, control; lane 5, LPS (10 μg/ml); lane 6, Hsp70 (10 μg/ml); lane 7, Hsp60 standard.

(PBMCs and Jurkat cells) the release of LDH was 0·95% and 1·03%, respectively (Table 1). The release of Hsp70 from cells under normal conditions was significantly greater in terms of the total cell contents ($P < 0·001$) than these rates (12·72% and 3·40%, respectively). The release of Hsp60 from these control cells was not significantly different from the percentage LDH release (0·85% and 1·66%, respectively) (Table 1). Only LPS caused a small increase in percentage LDH release in both cell types (Table 1). However, in all cases when Hsp70 and Hsp60 release was increased (to 20% or more) the LDH release remained below 3% (Table 1), indicating that cell damage was not responsible for the effects observed. Percentage cell viability as determined by Trypan blue showed no significant difference between controls and treated samples following 24 h incubation (data not shown). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay results for PBMCs and Jurkat indicated that 24 h treatment with Hsp60, Hsp70 and LPS had no effect on cell number/metabolic activity. GroEL caused a small, but significant, increase in cell number/metabolic activity (data not shown).

Following the observed release from PBMCs and Jurkat cells it was decided that a non-lymphocyte cell line would be investigated for effects of the same treatments. Hsp70 was released from GCT cells under normal cell culture conditions during 24 h cell culture at 37°C. Both LPS and Hsp60 inhibited ($P < 0·01$) Hsp70 release from GCT cells, while GroEL caused a large increase ($P < 0·001$) (Fig. 4). GCT osteoblast-like cells did not release any Hsp60 under control conditions or following treatment with LPS for 24 h (Fig. 4). Hsp60 release increased significantly ($P < 0·001$) following treatment with both GroEL and Hsp70.

Intracellular concentrations of Hsp60 and Hsp70 were measured following all treatments (Table 2). Hsp60 treatment resulted in a decrease in intracellular Hsp70 in PBMCs ($P < 0·05$) and GCTs ($P < 0·05$) (Table 2). GroEL resulted in a decrease in intracellular Hsp70 in all cells ($P < 0·05$) and intracellular Hsp60 in PBMCs ($P < 0·05$) (Table 2). The effect of LPS on intracellular Hsp70 concentration was an increase in PBMCs ($P < 0·05$) and a decrease in GCTs ($P < 0·05$) (Table 2). The effect of LPS on intracellular Hsp60 was to cause a decrease in PBMCs and Jurkat cells ($P < 0·05$) and an increase in GCTs ($P < 0·05$) (Table 2). Hsp70 resulted in an increase in intracellular Hsp60 in PBMCs ($P < 0·05$) and a decrease in Jurkat cells ($P < 0·01$) (Table 2).

Discussion

There is an increasing amount of evidence that Hsps have cytokine-like functions and may act as danger signals to the innate immune system [1–3,39,40]. If the role of Hsp release from cells is to act as a danger signal then it would seem reasonable that their release would be increased following exposure to bacterial infection. We have previously demonstrated

Table 1. Percentage release of heat shock protein (Hsp)70, Hsp60 and lactate dehydrogenase (LDH). Hsp70, Hsp60 and LDH were measured in culture media following incubation with lipopolysaccharide (LPS) (10 μg/ml), GroEL (10 μg/ml), Hsp60 (10 μg/ml) and Hsp70 (10 μg/ml) for 24 h. Percentage release was calculated from measurements of protein released and that present in cell extracts.

		% Release	
	Treatment	PBMC	Jurkat
Hsp70 release	Control	12·72 ± 0·82	3·40 ± 0·18
% total cell Hsp70	LPS	19·16 ± 0·74	29·14 ± 1·47
$n = 3$	GroEL	71·35 ± 1·98	24·18 ± 5·11
	Hsp60	8·78 ± 0·15	3·97 ± 0·27
Hsp60 release	Control	0·85 ± 0·13	1·66 ± 0·24
% total cell Hsp60	LPS	20·27 ± 1·96	2·91 ± 0·13
$n = 3$	GroEL	33·66 ± 2·04	5·99 ± 0·48
	Hsp70	59·22 ± 0·49	26·93 ± 1·39
LDH release	Control	0·95 ± 0·19	1·03 ± 0·28
% of total cell LDH	LPS	1·87 ± 0·38	2·74 ± 0·83
$n = 3$	GroEL	1·22 ± 0·27	1·81 ± 0·40
	Hsp60	1·67 ± 0·79	0·87 ± 0·08
	Hsp70	2·06 ± 0·18	1·87 ± 0·39

PBMC: peripheral blood mononuclear cells.

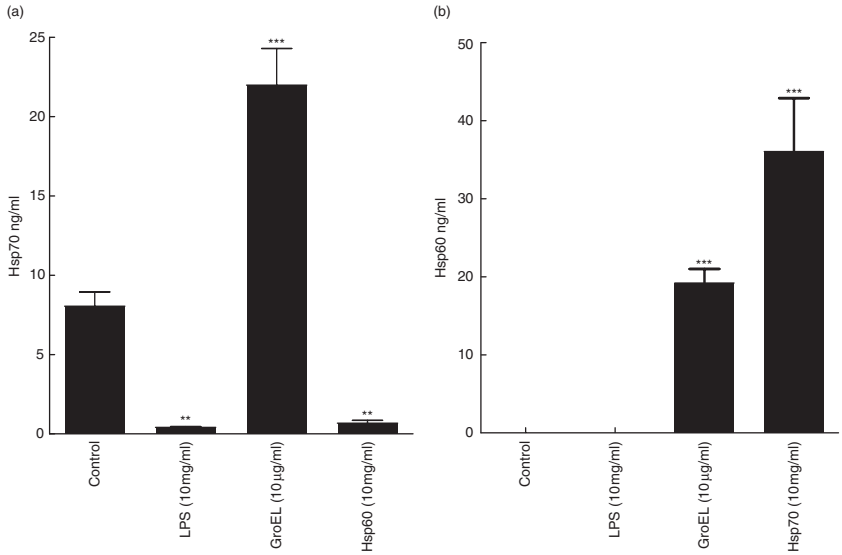

Fig. 4. Heat shock protein (Hsp)70 (a) and Hsp60 (b) release into culture media from giant cell tumour (GCT) cells following 24 h treatment with lipopolysaccharide (LPS) (10 μg/ml), GroEL (10 μg/ml), Hsp60 (10 μg/ml) and Hsp70 (10 μg/ml). Data presented as mean ± s.e.m., $n = 3$. Indicates significant difference from 37°C control; $^{**}P = < 0{\cdot}01$; $^{***}P = < 0{\cdot}001$.

release of Hsp70 from PBMCs [36]. The aim of this work was to investigate whether Hsp70 and/or Hsp60 release from cells could be up-regulated following exposure to LPS, GroEL or human Hsps.

Hsp70 was released by PBMCs, a T cell line (Jurkat) and a primary GCT when cultured at 37°C for 24 h. Under these conditions the release of Hsp60 was not significantly greater than the release of LDH from cells in percentage terms. These data suggest that under normal conditions the cells are releasing Hsp70, but not Hsp60. In general, Hsp60 and Hsp70 release from PBMCs and Jurkat cells was found to be increased following exposure to LPS or GroEL. Human Hsp70 also stimulated Hsp60 release from these cells. Cell viability counts and LDH measurements established that the responses observed were not due to cellular damage and Western blots confirmed the release of complete Hsp60 and Hsp70 proteins. The intracellular concentrations are presented in Table 2.

LPS caused a significant increase in Hsp70 and Hsp60 release from PBMCs and Hsp70 release from Jurkat cells. However, LPS did not affect Hsp60 release from Jurkat or GCT cells and inhibited Hsp70 release from GCT cells. One pathway through which LPS is known to affect cells is via CD14/TLR4 or TLR2 receptors [41]. CD14 receptors are not present on T cells and are not normally present on osteoblasts [42]; the response of Hsp60 release to LPS follows this CD14 pattern of expression. The differences between the effects of LPS on Jurkat and GCT cells suggest that there are also differences in the signalling pathways leading to Hsp70 or Hsp60 release. Although GroEL decreased the intracellular concentrations in several treatments, these decreases are not sufficient to account for the increases in release. The suggestion that Hsp60 and Hsp70 release are regulated processes, involving gene expression and membrane transport rather than simply the result of mass flow, is supported by the intracellular data.

A response identified in all cell lines (immune and non-immune) was that of elevated Hsp70 release in response to GroEL, but not to human Hsp60. These patterns of Hsp release suggested that there are different receptors and/or responses to human and bacterial Hsp60. This is supported further by the difference between the stimulation of Hsp60 release by Hsp70 and GroEL. Responses to GroEL have been shown to be species specific within bacteria [43] and microbial and mammalian Hsp60 compete for different binding sites [44]. These differences are consistent with the Hsp danger signal hypothesis.

We have therefore demonstrated that Hsp70 and Hsp60 release can be up-regulated by exposure to bacterial antigens. We have shown previously that Hsp70 is released from both B and T lymphocytes [36] and we suggest that both these cell

Table 2. Intracellular heat shock protein (Hsp)70 and Hsp60 from peripheral blood mononuclear cells (PBMCs), Jurkat cells and giant cell tumour cells (GCTs) following incubation with lipopolysaccharide (LPS) (10 μg/ml), GroEL (10 μg/ml), Hsp70 (10 μg/ml) and Hsp60 (10 μg/ml) for 24 h.

	PBMC ng/10^6 cells	Jurkat ng/10^6 cells	GCT ng/ml
Hsp70			
Control	1·50 ± 0·08	19·61 ± 0·30	21·40 ± 0·78
Hsp60	1·03 ± 0·04*	16·45 ± 0·18	15·65 ± 0·06*
GroEL	1·08 ± 0·07*	13·00 ± 0·35*	11·71 ± 1·06*
LPS	2·93 ± 0·04	15·57 ± 1·69	10·21 ± 1·30*
Hsp60			
Control	3·66 ± 0·08	58·32 ± 3·77	22·69 ± 2·09
Hsp70	5·25 ± 0·09*	40·42 ± 1·19**	29·36 ± 1·34
GroEL	2·03 ± 0·19*	51·64 ± 3·01	33·90 ± 1·94*
LPS	3·53 ± 0·02*	43·59 ± 2·14*	35·35 ± 0·27*

Data presented as mean ± s.e.m., $n = 3$. *Indicates significant difference from 37°C control; $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

types are probably involved in the responses to bacterial antigens observed in this study. Confirmation of the role of each cell type in this response is worthy of further study. Hsp70 and Hsp60 have been demonstrated to be capable of activating monocytes, macrophages and dendritic cells [11,45], and also of inducing secretion of a wide range of cytokines [11,13,18,20,39]. Hsp70 has also been shown here to stimulate Hsp60 release in all cells tested. Conversely, Hsp60 had no effect on Hsp70 release in Jurkat cells, but inhibited release in PBMCs and GCT cells. Overall, these results, combined with those on the bacterial antigens, indicate different receptors and pathways are involved in the responses [20,21].

Although Hsps and LPS have been demonstrated to act through a common pathway involving CD14 [13,15,18,39] there are reports that some responses of cells to recombinant Hsps are due to LPS contamination [46,47]. The methods adopted in this paper have taken the possibility of LPS contamination into consideration. Hsp preparations were treated with polymyxin B or boiled at 100°C for 10 min. The effects on Hsp release from Jurkat cells were unaffected by the former and completely removed by the latter (data not shown). The Hsp70 preparations used in these experiments have been shown to contain LPS [47]. We have repeated experiments on Jurkat cells with the low LPS Hsp preparations (Hsp60, ESP-540E; and Hsp70, ESP-555E, Stressgen). It is possible that the treatments described did not remove all LPS activity. However, we observed differential responses to LPS and the recombinant Hsps, and the responses to GroEL and Hsp70 were significantly greater than responses to LPS. Therefore, despite the fact that Hsp60 strongly binds LPS [48], we suggest that either LPS contamination cannot be totally responsible for the results obtained, or that the biological activity of LPS is altered when bound to the Hsp.

Bacterial infection causes an increase in serum Hsp70 [2]. We have presented evidence that bacterial antigens stimulate Hsp70 and Hsp60 release from cells of the immune system. The danger model requires a signalling system in response to pathogens [49,50]. The data presented here support the hypothesis that Hsp70 and Hsp60 form part of a danger-signalling cascade. HSPs have a dual role in such a system; first, as bacterial antigens molecules such as GroEL (as well as LPS) are recognized as non-self and the immune system reacts. One of these reactions, described in this paper, is the release of Hsp60 and Hsp70. These proteins have differential effects on the cells of the immune system and can therefore be considered immunoregulatory. There are potential consequences of this signalling role of Hsps in other situations where Hsps are elevated in serum, such as exercise [29] and psychological stress [2].

Many questions remain regarding the signalling pathways and receptors involved in the observed responses, although it is clear that the release observed to this point could have important implications for demonstrating the role of Hsps as chemokines and for their causative and protective roles in disease processes.

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