TNF- α , IL-1 α , IL-6 and ICAM-1 expression in human keratinocytes stimulated *in vitro* with *Escherichia coli* heat-shock proteins

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Bacterial heat-shock proteins (HSPs) from *Escherichia coli* (GroES, GroEL and DnaK) were studied for their ability to induce by themselves the expression and release of tumour necrosis factor- α (TNF- α), interleukin-1 α (IL-1 α), interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1) by cultured human keratinocytes. The surface expression of ICAM-1 was also investigated. In the supernatants of untreated cells none or a minimal amount of these molecules was found. After 48 h of stimulation with GroEL significant amounts of TNF- α , IL-1 α , IL-6 and soluble ICAM-1 were detected, reaching maximum concentrations at 1 μ g ml⁻¹. The same effect was elicited by DnaK but to a lesser extent. Treatment of keratinocytes with GroEL and DnaK also increased TNF- α , IL-1 α , IL-6 and ICAM-1 mRNA levels. GroES showed significant activity only on the expression and release of IL-6. GroEL and DnaK were also able to up-regulate the surface expression of ICAM-1 on keratinocytes. The effects on ICAM-1 expression seemed to be directly due to HSPs and not mediated via cytokines. Furthermore, these effects were due to the properties of HSPs because they were inhibited by specific monoclonal antibodies. These findings support the potential role of HSPs in modulating cell interactions during immunological and inflammatory responses in the skin.

Keywords: heat-shock proteins, TNF- α , IL-1 α , IL-6, ICAM-1

INTRODUCTION

Heat-shock proteins (HSPs) are evolutionarily highly conserved polypeptides with important biological functions in protein biogenesis (Georgopoulos & Welch, 1993). They appear to be produced by prokaryotic and eukaryotic cells to preserve cellular functions under a variety of stress conditions, including heat-shock, nutrient deprivation, and viral and bacterial infections (Fayet *et al.*, 1989; Kaufmann, 1990; Lindquist & Craig, 1989; Pelham, 1988). These proteins are predominantly located in intracellular compartments but recent evidence suggests that some HSPs can be expressed on the cell surfaces and secreted extracellularly (Ensgraber & Loss, 1992).

Recently, it has been shown that *in vitro* exposure of human monocytes to some HSPs induces the release of

proinflammatory cytokines, such as tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) (Georgopoulos & Welch, 1993; Retzlaff *et al.*, 1994; Zhang *et al.*, 1993; Peetermans *et al.*, 1995a), but little is known about the role of these proteins in the host protective response.

It is well-known that the mycobacterial HSP 65, which belongs to the major 60 kDa HSP family that displays a high degree of genotypic and phenotypic homology among various micro-organisms, is an immunodominant antigen able to elicit a strong humoral and cellular immune response (Peetermans *et al.*, 1995a). HSP 65 homologues are abundantly expressed in inflammatory lesions and produced by micro-organisms during invasive infection and phagocytosis (Buchmeier & Heffron, 1990; Peetermans *et al.*, 1995b).

Our hypothesis is that HSPs are involved in the pathogenesis of various inflammatory diseases and add to a first-line host defence against invasive pathogens by their ability to induce the release of proinflammatory

Abbreviations: HSP, heat-shock protein; ICAM-1, intercellular adhesion molecule-1; IL-1 α , interleukin-1 α ; IL-6, interleukin-6; LDH, lactate dehydrogenase; sICAM-1, soluble ICAM-1; TNF- α , tumour necrosis factor- α .

cytokines, which in turn activate antimicrobial functions of mononuclear phagocytes. The aim of this study was to assess whether different HSPs from *Escherichia coli* are able to induce TNF- α , interleukin-1 α (IL-1 α), interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1) expression and release by keratinocytes.

Epidermal cells, and in particular keratinocytes, have been recently recognized to play a central role in transmitting proinflammatory signals by secreting cytokines and growth factors (Luger & Schwarz, 1990). The constitutive production of these factors, in particular IL-1 and TNF- α , is usually very low, but can be induced significantly by various injurious agents such as endotoxin, virus particles, tumour promoters or UV light (Luger & Schwarz, 1990; Kupper, 1990; Köch *et al.*, 1990). In the case of human keratinocytes, both IL-1 α and IL-1 β are expressed, although IL-1 α is the major secreted isotype (Kupper, 1990).

IL-1 and TNF- α in their turn act with an autocrine mechanism on keratinocytes, inducing the release of a cascade of other cytokines which can have a pro-inflammatory or chemotactic activity (IL-6, IL-8, granulocyte-monocyte colony stimulating factor) and the expression of adhesion molecules, in particular ICAM-1 (Shalaby *et al.*, 1984; Dinarello *et al.*, 1986; Sauder *et al.*, 1988; Groves *et al.*, 1992; Trefzer *et al.*, 1991; Nickoloff *et al.*, 1991). ICAM-1 is expressed by a wide variety of cells (Dustin *et al.*, 1986) and is able to initiate cellular interactions in the immune system.

The constitutive expression of ICAM-1 by keratinocytes is minimal but can be stimulated by several cytokines (interferon- τ , TNF- α). Furthermore, recent studies have demonstrated that a soluble form of ICAM-1 (sICAM-1) is released during inflammatory reactions (Seth *et al.*, 1991).

These molecules are not only involved in the mediation of local inflammatory reactions but also cause systemic effects. Thus it is of great interest to explore the various factors, especially bacterial components, able to induce in keratinocytes the expression and release of molecules which show biological activities, not only for the understanding of some skin diseases, but also to gain further insight into the role played by these cells in inflammatory and immune responses.

METHODS

***E. coli* HSPs.** GroES, the 10 kDa HSP (HSP 10) of *E. coli*, GroEL, the 60 kDa HSP (HSP 60) of *E. coli*, and DnaK, the 70 kDa HSP (HSP 70) of *E. coli*, were purchased from Boehringer Mannheim.

Keratinocyte cell cultures. Surgical foreskin specimens, obtained from young patients, were rinsed in PBS (0.145 sodium chloride, 0.15 sodium phosphate) with 10% penicillin/streptomycin and 1% amphotericin B for 30 min, washed in PBS and then incubated overnight in 1% dispase II (Boehringer Mannheim, 0.5 U mg⁻¹) at 4 °C to cause epidermal separation. Epidermal sheets were treated with 0.25% trypsin containing 0.34 M EDTA. After 20 min incubation at 37 °C under gentle agitation, the cells were centrifuged and resuspended in serum-

free keratinocyte medium supplemented with recombinant epidermal growth factor (5 ng ml⁻¹) and bovine pituitary extract (50 µg ml⁻¹) (Gibco). The keratinocytes were plated into sterile 6-well plates and grown at 37 °C 5% CO₂. Cells were trypsinized and replated into 250 ml tissue culture flasks until primary cultures reached confluence. Cultures at their third or fourth passage were used in all subsequent studies (Walev *et al.*, 1993). Immunofluorescent staining using monoclonal antibodies to keratins (Boehringer Mannheim), to Langerhans cells (OKT6, Ortho Pharmaceuticals), to melanocytes (S-100, Ortho), to lymphocytes (OKT3, Ortho) and to major histocompatibility complex class II antigen (HLA-DR) (OKDR, Ortho) verified that these cell cultures consisted of 100% keratinocytes and did not express HLA-DR antigen.

Induction of cytokine and sICAM-1 production. Keratinocytes (3 × 10⁶ ml⁻¹) were incubated at 37 °C in 5% CO₂ for up to 48 h either with HSPs diluted in the cell culture at the appropriate concentrations (0.05–5 µg/ml) or with *E. coli* serotype O26:B6 LPS (1 µg ml⁻¹, Sigma). Pilot studies suggested that 48 h was the average release time. After contact, the culture supernatants were harvested for cytokine and sICAM-1 assays. Cell viability was then checked by the Trypan blue exclusion test. Cell stimulation was also performed with trypsinized HSPs. To this end, proteins were cleaved overnight at room temperature with immobilized trypsin (treated with *N*-tosyl-L-phenylalanine chloromethyl ketone and attached to beaded agarose; Sigma) (Koga *et al.*, 1989).

Cytokine and sICAM-1 assays. All measurements were carried out by using monoclonal antibodies. TNF- α , IL-6, IL-1 α and ICAM-1 were measured by immunoassays (TNF- α - and IL-6-ELISA, Biochrom; IL-1 α Quantikine, R & D Systems; Soluble ICAM-1 ELISA kit, British Bio-technology).

Lactate dehydrogenase (LDH) assay. This was carried out according to the manufacturer's instructions using a Cytotoxicity Detection kit (Boehringer Mannheim). LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into the cell culture supernatant upon damage of the plasma membrane. LDH activity was determined by a coupled enzymic reaction whereby the tetrazolium salt (INT) was reduced to formazan. An increase in the number of dead or damaged cells resulted in an increase in LDH activity in the culture supernatant.

RNA isolation and cDNA preparation. Human keratinocytes (1 × 10⁷), cultured in complete medium under different experimental conditions, were collected after 3 h and total RNA was extracted according to Chomczynski & Sacchi (1987). The RNA pellet was resuspended in 75% ethanol, sedimented, vacuum-dried and dissolved in 15 µl RNase-free water. One µg of oligo(dT) (Promega Biotec) was added to the suspension and the mixture was heated at 65 °C for 5 min. After cooling on ice the mixture was incubated for 2 h at 42 °C with 14 µl of the following mixture: 20 mM DTT (Sigma); 1 mM (each) dATP, dGTP, dCTP and dTTP; 35 U RNasin (Promega); and 525 U of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) in reverse transcription buffer (Gibco BRL).

PCR procedure. PCR primers for human TNF- α , IL-1 α , IL-6 and β actin were purchased from Clontech. Human ICAM-1 primer pair sequences were designed on the basis of published gene sequences as follows: sense, 5'-CGTGCCGCACTGAACTGGAC-3'; anti-sense, 5'-CCTCACACTTCACTGTCACTCT-3'. The primer sequences were complementary to sequences in the exons or spanned exon-exon junctions and

thus were RNA-specific. Two microlitres of cDNA, prepared as described above, was amplified in the presence of 500 nM (final concentration) 5' and 3' primers, 200 µM (each) dATP, dGTP, dCTP and dTTP, and 1.25 U *Taq* DNA polymerase (Promega) in a final volume of 50 µl *Taq* DNA polymerase 10 × buffer (Promega). The PCR was performed in a Perkin-Elmer thermal cycler for 30 cycles as follows: 1 min denaturation at 94 °C, 2 min annealing at 60 °C and 3 min extension at 72 °C. The reaction product was visualized by electrophoresis using 25 µl of the reaction mixture at 100 V in 1.5 % agarose gel containing ethidium bromide (1 µg ml⁻¹). The gels were then examined on a UV light box and photographed. One microgram of *Bgl*I- and *Hinf*I-digested pBR328 DNA (Boehringer Mannheim) was run in parallel as a molecular mass marker (providing bands at 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 bp).

PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples for each experiment; data shown are representative of at least three different experiments.

Up-regulation of membrane ICAM-1. Keratinocytes (1×10^6) were plated in 6-well tissue culture plates and incubated at 37 °C overnight. The different HSPs were added 12, 24 and 48 h prior to staining the cells for ICAM-1.

After incubation at 37 °C for different lengths of time, cells were rinsed twice with PBS, harvested by trypsinization and then processed for immunofluorescence analysis.

Previous experiments established that this method of cell harvesting does not affect the surface expression of any of the antigens studied when compared to non-enzymically harvested cells.

Surface ICAM-1 expression by keratinocytes was determined by indirect immunofluorescence. Collected and washed cells were incubated with a 1:40 dilution of the mouse anti-human ICAM-1/CD54 monoclonal antibody (Boehringer Mannheim) for 30 min at 4 °C. Cells were then washed three times in PBS containing 0.05 % sodium azide, resuspended and incubated with a 1:20 dilution of fluorescein-isothiocyanate-conjugated goat anti-mouse IgG (Sigma). Subsequently, cells were washed three times and analysed using a Becton Dickinson FACS analyser (Becton Dickinson). Usually 5000 cells per sample were assessed and data are given as percentage ICAM-1-reactive cells in comparison to unstimulated control cells.

Blocking experiments. To verify whether the effects observed were due to the properties of HSPs they were inhibited by available specific monoclonal antibodies, which were added (0.5 µg ml⁻¹) to cell culture media immediately following stimulation by HSPs in each series of experiments. With this aim, monoclonal antibody anti-DnaK (clone 8E2) and anti-GroEL (clone 9A1) (Stressgene Biotechnologies) were used. To assess whether the expression of ICAM-1 is a direct or indirect effect due to HSPs, monoclonal antibody anti-human TNF-α, anti-human IL-1α and anti-human IL-6 obtained from Genzyme were used. To block ICAM-1 cytokine induction, the antibodies (0.5 µg ml⁻¹) were added to cell culture media immediately following stimulation by bacterial components. The keratinocytes were incubated for the usual length of time before the amounts of cell surface and released ICAM-1 were assayed.

Statistics. All experiments were carried out in triplicate; results are expressed as means ± SD. Comparisons between

tests were done by the Student's *t*-test, with statistical significance considered to be $P < 0.05$.

RESULTS

Purity of HSP preparations

All possible traces of LPS were identified by the *Limulus* amoebocyte lysate assay. This was performed with samples of each bacterial HSP and compared with standard LPS solution which was *Limulus*-amoebocyte-lysate-positive at 0.1 EU ml⁻¹ (14 pg ml⁻¹). (QCL-1000 Quantitative Chromogenic LAL, Biowhittaker). All HSPs were negative in the *Limulus* amoebocyte lysate test at the concentrations used.

In addition, to neutralize the biological activity of traces of LPS that could be present in the preparation, HSPs were incubated with polymyxin B (Sigma) at room temperature for 1 h at a ratio of 1:10. In all of the tests performed, HSPs plus polymyxin B gave the same results as HSPs alone (data not shown).

Furthermore, because the cytokine-stimulating activity of HSPs is heat sensitive, HSPs were first inactivated by heating for 1 h at 90 °C and then added to cell cultures. Heated HSPs had no effect on the expression and release of cytokines and ICAM-1 whereas LPS was fully active under the same experimental conditions (data not shown).

TNF-α release

Stimulation with some HSPs caused the appearance of TNF-α in the supernatant of keratinocyte cultures (Fig. 1a). Control cells incubated for 48 h revealed no significant release of TNF-α. As expected, stimulation with LPS (1 µg ml⁻¹) induced a marked release of TNF-α. Under our experimental conditions, TNF-α release was also induced by GroEL and DnaK extracted from *E. coli*, at concentrations ranging from 0.1 to 5 µg ml⁻¹. The quantity of TNF-α released increased progressively up to GroEL and DnaK concentrations of 1 µg ml⁻¹. Concentrations higher than this had no further effect on the production of TNF-α and concentrations lower than 0.1 µg ml⁻¹ GroEL and DnaK showed no significant effect. TNF-α release obtained using GroEL as a stimulus was similar to that induced by LPS at a concentration of 1 µg ml⁻¹, while keratinocytes stimulated with DnaK showed a lower release. GroES had no significant effect on the release of TNF-α under the same experimental conditions. The release of TNF-α by keratinocytes stimulated with GroEL and DnaK was already detectable after 12 h incubation and reached its highest level after 48 h incubation (Table 1).

IL-1α release

The release of IL-1α by cultured keratinocytes stimulated with different HSPs is shown in Fig. 1(b). Controls incubated for 48 h revealed no significant amount of IL-

Fig. 1. Release of TNF- α (a), IL-1 α (b), IL-6 (c) and sICAM-1 (d) from human keratinocytes incubated for 48 h with GroES (□), GroEL (▨) and DnaK (■) at different concentrations and with LPS (1 $\mu\text{g ml}^{-1}$) (▤). Results obtained with controls incubated without HSPs or LPS are also shown (▤). Data are means of three experiments $\pm$ SD. Asterisks indicate statistically significant differences ($P < 0.05$) versus untreated cells.

1 α release, while LPS-stimulated cells released high levels of IL-1 α . IL-1 α release was also induced by GroEL and DnaK at concentrations ranging from 0.1 to 5 $\mu\text{g ml}^{-1}$. The quantity of IL-1 α released increased progressively up to GroEL and DnaK concentrations of 1 $\mu\text{g ml}^{-1}$. Thus the greatest release was obtained using the same concentrations that most effectively induced TNF- α release. Concentrations higher than 1 $\mu\text{g ml}^{-1}$ GroEL and DnaK had no further effect on the production of IL-1 α and concentrations lower than 0.1 $\mu\text{g ml}^{-1}$ showed no significant effect. Keratinocytes treated with GroEL and DnaK were able to release IL-1 α in amounts similar to those obtained using LPS (1 $\mu\text{g ml}^{-1}$) as a stimulus. No effect was detected when cells were treated with GroES at concentrations ranging

from 0.05 to 5 $\mu\text{g ml}^{-1}$. When GroEL and DnaK were used as a stimulus, IL-1 α appeared in the supernatant after 12 h and reached its highest level after 48 h incubation (Table 1).

IL-6 release

All the stimuli used induced IL-6 release from keratinocytes *in vitro* (Fig. 1c). Controls incubated for 48 h revealed a slight IL-6 release. LPS (1 $\mu\text{g ml}^{-1}$) was a potent stimulus for IL-6 release. GroEL and DnaK were also able to induce significant IL-6 release starting at concentrations of 0.1 $\mu\text{g ml}^{-1}$ and reaching their maximum activity at 1 $\mu\text{g ml}^{-1}$. GroES was able to induce significant IL-6 release only at concentrations higher

Table 1. Kinetics of TNF- α , IL- α , IL-6 and sICAM-1 release by human keratinocytes stimulated with *E. coli* HSPs

Keratinocytes were treated for up to 48 h with GroES (1 $\mu\text{g ml}^{-1}$), GroEL (1 $\mu\text{g ml}^{-1}$) and DnaK (1 $\mu\text{g ml}^{-1}$). After 12, 24 and 48 h cell supernatants were harvested for cytokine and sICAM-1 assays. Data are means of three representative experiments $\pm$ SD.

Challenge		Time (h)			
		0	12	24	48
TNF- α concn (pg ml $^{-1}$)	GroES	90 $\pm$ 9.6	91 $\pm$ 9.7	95 $\pm$ 9.7	98 $\pm$ 9.8
	GroEL	92 $\pm$ 9.5	220 $\pm$ 14.8	760 $\pm$ 27.5	998 $\pm$ 31.6
	DnaK	89 $\pm$ 9.4	110 $\pm$ 10.5	385 $\pm$ 19.6	750 $\pm$ 27.3
IL-1 α concn (pg ml $^{-1}$)	GroES	6 $\pm$ 2.2	7 $\pm$ 2.6	11 $\pm$ 3.3	15 $\pm$ 3.8
	GroEL	5 $\pm$ 2.2	32 $\pm$ 5.6	135 $\pm$ 11.6	187 $\pm$ 13.6
	DnaK	6 $\pm$ 2.3	22 $\pm$ 4.6	128 $\pm$ 12.3	175 $\pm$ 13.7
IL-6 concn (pg ml $^{-1}$)	GroES	3 $\pm$ 1.5	5 $\pm$ 2.4	8 $\pm$ 2.7	16 $\pm$ 3.8
	GroEL	4 $\pm$ 2.1	15 $\pm$ 3.8	43 $\pm$ 6.5	52 $\pm$ 7.5
	DnaK	3 $\pm$ 1.3	7 $\pm$ 2.6	28 $\pm$ 5.3	43 $\pm$ 6.7
sICAM-1 concn (ng ml $^{-1}$)	GroES	0.2 $\pm$ 0.1	0.5 $\pm$ 0.3	0.7 $\pm$ 0.5	0.7 $\pm$ 0.4
	GroEL	0.4 $\pm$ 0.3	1.8 $\pm$ 0.8	3.2 $\pm$ 1.5	8.2 $\pm$ 2.5
	DnaK	0.3 $\pm$ 0.1	1.3 $\pm$ 0.7	1.9 $\pm$ 0.8	6.4 $\pm$ 2.1

than 1 $\mu\text{g ml}^{-1}$. GroEL and DnaK used at concentrations higher than 1 $\mu\text{g ml}^{-1}$ had no further effect on IL-6 release. The release of IL-6 induced by HSPs was in all cases lower than that obtained after stimulation with LPS. IL-6 release from keratinocytes stimulated with HSPs revealed time kinetics similar to those obtained for TNF- α and IL-1 α , showing the maximum level after 48 h incubation (Table 1).

sICAM-1 release

Of the stimuli assayed, LPS, GroEL and DnaK significantly increased the amount of sICAM-1 in the supernatant of cultured keratinocytes (Fig. 1d). Control cells incubated for 48 h showed no sICAM-1 release in the supernatants. GroEL and DnaK were able to induce significant sICAM-1 release starting at concentrations of 0.1 $\mu\text{g ml}^{-1}$ and reaching their maximum activity at 1 $\mu\text{g ml}^{-1}$. Keratinocytes stimulated with GroEL (1 $\mu\text{g ml}^{-1}$) released approximately the same amount of sICAM-1 as cells treated with LPS (1 $\mu\text{g ml}^{-1}$), while DnaK at the same concentration was able to induce a lower release. Treatment of keratinocytes with GroES at different concentrations (0.05–5 $\mu\text{g ml}^{-1}$) caused no significant production of sICAM-1. When cells were treated with GroEL and DnaK, the release of sICAM-1 began after 12 h and the maximum release occurred after 48 h of incubation (Table 1).

LDH assay

The release of TNF- α , IL-1 α , IL-6 and sICAM-1 was not due to cell lysis; LDH, as a cytoplasmic marker, was found to be present in the supernatant of stimulated cells at levels similar to those detected in the supernatant of unstimulated cells (data not shown).

TNF- α , IL-1 α , IL-6 and ICAM-1 mRNA expression in human keratinocytes stimulated with HSPs

Fig. 2 shows a representative gel containing the reverse-transcription-PCR products of RNA extracted from HSP-treated keratinocyte cultures. Cells were stimulated with the HSP concentrations that showed the best activity in inducing the release of TNF- α , IL-1 α , IL-6 and sICAM-1. The intensity of the signal from TNF- α , IL-1 α , IL-6 and sICAM-1 mRNA increased when cells were treated with GroEL and DnaK (1 $\mu\text{g ml}^{-1}$). Stimulation with GroEL and DnaK (1 $\mu\text{g ml}^{-1}$) induced a similar expression of IL-1 α and IL-6 mRNA, while GroEL was able to induce higher expression of TNF- α and ICAM-1 mRNA. Stimulation of keratinocytes with GroES (1 $\mu\text{g ml}^{-1}$) induced a good expression only of IL-6 mRNA. No effect on TNF- α , IL-1 α and ICAM-1 mRNA was detected when cells were treated with GroES at concentrations ranging from 0.05 to 5 $\mu\text{g ml}^{-1}$.

Up-regulation of ICAM-1 expression on keratinocyte surfaces

As detected by flow cytometry, constitutive expression of ICAM-1 was low but expression was stimulated by LPS (1 $\mu\text{g ml}^{-1}$), GroEL (1 $\mu\text{g ml}^{-1}$) and DnaK (1 $\mu\text{g ml}^{-1}$) (Table 2). The increase of ICAM-1 levels began within 12 h of treatment, and this up-regulation continued for up to 48 h. GroES showed no activity on ICAM-1 surface expression.

Blocking experiments

Monoclonal antibodies to TNF- α , IL-1 α and IL-6 did not significantly modify ICAM-1 expression and release

Fig. 2. Effect of HSPs on the steady-state levels of TNF- α , IL-1 α , IL-6 and ICAM-1 mRNA in human keratinocytes. Cultures were treated for 3 h either with culture medium (control), *E. coli* LPS ($1 \mu\text{g ml}^{-1}$) or *E. coli* HSPs ($1 \mu\text{g ml}^{-1}$) and then subjected to RNA extraction and reverse-transcription-PCR amplification. Reaction products were run on 1.5% agarose gels in the presence of appropriate molecular mass markers (arrow); β -actin was the positive transcription control.

in keratinocytes stimulated with LPS ($1 \mu\text{g ml}^{-1}$), GroEL ($1 \mu\text{g ml}^{-1}$) and DnaK ($1 \mu\text{g ml}^{-1}$) (data not shown). Monoclonal antibodies anti-DnaK and anti-GroEL inhibited the biological activities shown by HSPs under our experimental conditions (data not shown).

Cell stimulation with trypsinized HSPs

To verify whether the stimulating activity of HSPs was protease-sensitive, all the tests were also performed using trypsinized HSPs as a stimulus. HSPs showed no biological activities under this experimental condition (data not shown).

Table 2. ICAM-1 surface expression after treatment with *E. coli* HSPs

Keratinocytes were treated for 12, 24 and 48 h with LPS ($1 \mu\text{g ml}^{-1}$), GroES ($1 \mu\text{g ml}^{-1}$), GroEL ($1 \mu\text{g ml}^{-1}$) and DnaK ($1 \mu\text{g ml}^{-1}$). ICAM-1 surface expression was assessed by flow cytometry of 5000 cells per data point as described in Methods. Data are given as percentage ICAM-1-reactive cells in comparison to unstimulated control cells and are means of three representative experiments $\pm$ SD.

Challenge	Time (h)		
	12	24	48
None	2 ± 1	3 ± 1	3 ± 1
LPS	18 ± 5	29 ± 6	27 ± 4
GroES	3 ± 1	5 ± 2	4 ± 1
GroEL	11 ± 3	24 ± 5	22 ± 4
DnaK	8 ± 2	19 ± 3	17 ± 4

DISCUSSION

The HSPs, one of the most highly conserved group of proteins, are implicated as essential components in a number of different biological processes distinct from protein processing.

However, the role played by HSPs during the host response has not been yet clarified. A wide variety of stimuli such as temperature (Lindquist & Craig, 1989), oxygen radicals (H_2O_2) (Clerget & Polla, 1990) and cytokines (Gromkowski *et al.*, 1989) are able to increase the synthesis and production of HSPs. It is likely that, at the inflammatory site, lysis of bacterial cells may cause HSP accumulation. These proteins, by themselves or together with other molecules which show biological activities, could interact with host cells involved in the specific and non-specific responses affecting their functions (Karlsson-Parra *et al.*, 1990; Polla, 1988; Kaufmann *et al.*, 1991). Therefore, in this work we evaluated the effect of *E. coli* HSPs with different molecular masses on the expression and release of some cytokines (TNF- α , IL-1 α and IL-6) and of one adhesion molecule (ICAM-1) in human keratinocytes.

In the inflammatory reaction of the skin, keratinocytes play a determining role by synthesizing and secreting cytokines and adhesion molecules (Koch *et al.*, 1990; Partridge *et al.*, 1991; Singer *et al.*, 1989). Cytokines have pleiotropic effects on a wide range of cell types, and influence keratinocytes by modifying the secretion pattern of cytokines and other growth factors (Luger & Schwarz, 1990; Arai *et al.*, 1990). Moreover, the adhesion molecules select the blood cells that are in turn activated locally, amplifying and modulating the inflammatory response by secreting soluble factors (Shalaby *et al.*, 1984; Marlin & Springer, 1987).

Our study demonstrated that among HSPs, GroEL and, to a lesser extent DnaK, were able to induce the release

of TNF- α , IL-1 α , IL-6 and sICAM-1 from keratinocytes at concentrations ranging from 0.1 to 5 $\mu\text{g ml}^{-1}$, with a maximum activity at 1 $\mu\text{g ml}^{-1}$. GroES showed significant activity only on the expression and release of IL-6.

The release of these molecules was not due to cell lysis; LDH, as a cytoplasmic marker, was found to be present in the supernatant of stimulated cells at levels similar to those detected in the supernatant of unstimulated cells (data not shown). Keratinocytes also exhibited an increase in ICAM-1 surface expression after treatment with GroEL and DnaK. Our results show that surface expression of ICAM-1 on keratinocytes treated with GroEL and DnaK began to increase after 12 h, reaching maximum levels after 24 h stimulation. All these findings were confirmed by reverse-transcription-PCR analysis of total RNA extracted from keratinocytes treated with the *E. coli* HSPs.

The effects on ICAM-1 expression seem to be directly due to HSPs and not mediated via cytokines because monoclonal antibodies anti-human TNF- α , anti-human IL-1 α and anti-human IL-6, used to block ICAM-1 cytokine induction, did not modify the adhesion surface expression induced by HSP treatment.

The main conclusion of this study is that DnaK and GroEL by themselves induce activation of human keratinocytes as demonstrated by their ability to express and release cytokines and ICAM-1.

These effects must be due to the properties of HSPs since they are inhibited by specific monoclonal antibodies. Furthermore, trypsinized HSPs showed no biological activities.

The effects of HSPs cannot be due to LPS contamination. Control experiments carried out using polymyxin B to neutralize traces of LPS that could be present in HSP preparations gave the same results as HSPs used alone. In addition, we performed all the tests using heat-inactivated HSPs. HSPs lost their activity under this experimental condition, while LPS was fully active.

The mechanisms involved in this process are not clear, but a possibility is the binding of HSPs to cell surface receptors. In fact, it is well-known that HSPs have a high affinity for immature or partially denatured proteins, binding them for the process of protein folding and unfolding (Hemmingsen *et al.*, 1988). The reason for the relatively lower potency of GroES in comparison with the other HSPs is not clear. An explanation might be related to the relatively small size of this protein (10 kDa) which prevents it from effectively cross-linking receptors following binding.

Our findings also suggest that HSPs may play an important role during inflammatory responses regulating the expression of molecules involved in the activation and adhesion of leukocytes.

The cytokines studied, along with sICAM-1, stimulate and select the cells involved in the immune response, such as T-cells and B-cells. Consequently, by releasing such factors, keratinocytes may intervene in regulating

the local immune response. A higher release of such factors, especially TNF- α , can be locally toxic to the host, which is best demonstrated by the crucial role of TNF- α in the pathogenesis of endotoxic shock in sepsis (Cerami & Beutler, 1988). In addition to a number of cytokines and growth factor receptors (Kohno *et al.*, 1990), soluble forms of adhesion molecules can also be released; L-selectin is released from the surface of neutrophils in response to a chemotactic stimulus (Smith *et al.*, 1991) and P-selectin is released from platelets (Dunlop *et al.*, 1992).

The potential function of soluble forms of adhesion molecules is at present the subject of speculation. Release from the cell surface may be a mechanism for down-regulating cytokine-induced increases in expression. Soluble molecules may function as competitive inhibitors of membrane-bound forms, thereby regulating cell adhesion. This has been demonstrated for a soluble form of P-selectin which is able to inhibit the adhesion of activated neutrophils to endothelial cells (Gamble *et al.*, 1990). Soluble forms may have cytokine-like activity. Evidence to support such a role comes from the demonstration that recombinant soluble E-selectin can act as a chemoattractant (Lo *et al.*, 1991). In addition, surface-bound ICAM-1 is able to have co-stimulatory effects on T-cells (Damle & Aruffo, 1991) and it is possible that soluble forms may also have a cell-signalling role.

Taken together, our results support the crucial role of HSPs in the pathogenesis of both local and systemic responses of the skin against micro-organisms. Further investigations on effector and regulatory functions of HSPs in other host and microbial systems should clarify the mechanisms involved in host-parasite relationships.

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