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Cytokine and Adhesion Molecule Expression in Human Monocytes and Endothelial Cells Stimulated with Bacterial Heat Shock Proteins

MASSIMILIANO GALDIERO,[1]* GABRIELLA CIPOLLARO DE L'ERO,[2] AND ANTONELLA MARCATILI[2]

Dipartimento di Patologia, Profilassi ed Ispezione degli Alimenti, Sezione Malattie Infettive, Facoltà di Veterinaria, Università degli Studi di Napoli Federico II,[1] and Istituto di Microbiologia, Facoltà di Medicina e Chirurgia, Seconda Università degli Studi di Napoli,[2] Naples, Italy

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Bacterial heat shock proteins (HSPs) from *Escherichia coli* (GroES, GroEL, and DNAk) were tested for their ability to induce by themselves the expression and release of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and granulocyte-monocyte colony-stimulating factor (GM-CSF) by human monocytes and GM-CSF, IL-6, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) by human umbilical vein endothelial cells (HUVEC). Our study demonstrated that treatment of monocytes with DNAk increased IL-6, TNF-α, and GM-CSF release in a dose-dependent manner. The same effect was elicited by GroEL but at a lower rate. Treatment of HUVEC cultures with DNAk and GroEL also increased GM-CSF, IL-6, E-selectin, ICAM-1, and VCAM-1 release in a dose-dependent fashion. In any case, the greatest release was obtained by using DNAk and GroEL at a concentration of 1 μg/ml. DNAk and GroEL were also able to up-regulate the surface expression of E-selectin, ICAM-1, and VCAM-1. As detected by reverse transcription-PCR analysis, DNAk and GroEL also increased the steady-state levels of cytokines and adhesion molecules in human monocytes and endothelial cells. In our study GroES showed a significant activity only on the release, surface expression, and mRNA transcription of E-selectin. Adhesion molecule expression seems to be a direct effect of HSPs and not via cytokines. Furthermore, these effects are due to HSPs properties because they are inhibited by specific monoclonal antibodies. These findings support the potential role of HSPs in modulating cell interactions during immunological and inflammatory responses.

Heat shock proteins (HSPs) are constitutively present in eukaryotic and prokaryotic cells with important biological functions in protein biogenesis (12). The production of HSPs is greatly enhanced by stress stimuli such as a rise in temperature, exposure to toxic oxygen radicals, nutritional deficiencies, and viral and bacterial infections (8, 17, 21, 27). The main function of these proteins is preservation of essential cellular proteins and functions. 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 (6, 7, 15). The ubiquitous distribution of these proteins and their high sequence conservation pose interesting challenges to the immune system. On the one hand, the omnipresence of epitopes shared by all phatogens may provide the immune system with a universal signal for infection. On the other hand, epitopes shared by the microorganism and the host may also furnish a link between infection and autoimmune diseases (4, 30).

HSPs are abundantly expressed in inflammatory lesions and produced by microorganisms during invasive infection and phagocytosis (2, 16, 25, 28). Although recent studies have demonstrated that some HSPs increase the production of monokines (9, 20, 26, 29, 33, 35), little is known about the role of these proteins in the host immune response. HSPs are likely involved in the pathogenesis of various inflammatory diseases and could add to a first-line host defense against invasive pathogens by their capacity to induce the endogenous production of proinflammatory cytokines, which in turn activate antimicrobial functions of mononuclear phagocytes (18, 19, 29). Human monocytes, generally, respond to microbial infections by increasing the expression and secretion of cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and granulocyte-monocyte colony-stimulating factor (GM-CSF) (20, 24, 32, 33), which are involved in inflammatory responses. On the other hand, it is well-known that endothelial cells contribute significantly to the pathophysiology of bacterial infections releasing adhesion molecules and cytokines (1). The microvascular endothelium plays an important role in regulating the exchange of fluids, macromolecules, and cells between the blood and the extravascular tissues. Endothelial cell injury contributes significantly to the patophysiology of bacterial sepsis and endotoxic shock (5, 23).

Besides the display of aggression by bacteria that invade the bloodstream, the endothelium can be lesioned by products of bacterial lysis, including HSPs. These products act locally on vessels and therefore are involved in the inflammatory response. Some bacterial products can be reabsorbed and enter circulation, where they act on large vessel endothelia.

Monocytes and endothelial cells play an important role in acute inflammatory processes. In fact they release cytokines, which are able to stimulate leukocyte functions and increase endothelial cell surface expression of molecules that support the adhesion of blood leukocytes (1). Therefore, the purpose of this study was to examine whether different HSPs from *Escherichia coli* are able to induce cytokine and adhesion molecule expression in human monocytes and endothelial cells.

* Corresponding author. Mailing address: Dipartimento di Patologia, Profilassi ed Ispezione degli Alimenti, Sezione Malattie Infettive, Facoltà di Veterinaria, Università degli Studi di Napoli Federico II, Via Federico Delpino, 1, 80137 Napoli, Italy. Phone: 01139-81-459560. Fax: 01139-81-458683.

MATERIALS AND METHODS

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

Cell preparation and culture. (i) Monocyte isolation. To exclude variability, leukocyte populations were obtained for each test series from a buffy coat of a

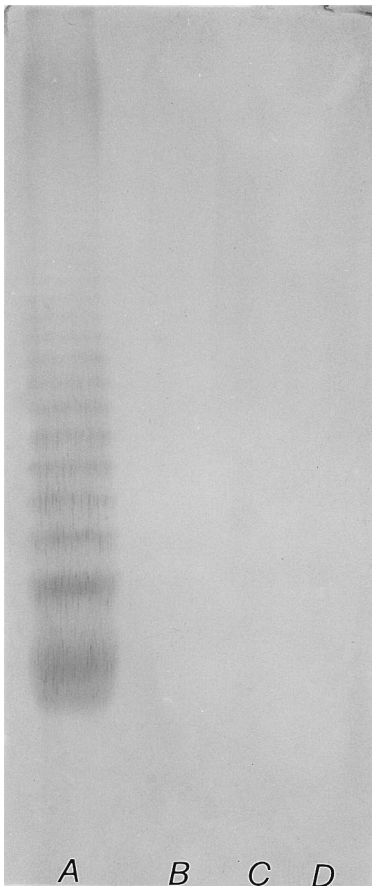

FIG. 1. Silver-stained SDS-PAGE of HSPs. Lane A, *Salmonella typhimurium* LPS standard (Difco); lane B, DNAk (10 μg); lane C, GroEL (10 μg); lane D, GroES (10 μg).

blood sample from a consenting healthy volunteer (courtesy of the Blood Bank, Second University of Naples). Buffy coat was diluted sixfold with RPMI 1640 (Labtek Laboratories, Eurobio, Paris, France) and washed twice. The pellet was resuspended in RPMI, applied on Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden), and centrifuged for 30 min at 900 × *g*. Peripheral blood mononuclear cells were washed twice and subsequently fractionated by centrifugation over continuous Percoll (Sigma Chemical Co., St. Louis, Mo.) gradients (4×10^8 cells/tube) at 500 × *g* for 30 min. The cell fraction enriched with monocytes was collected, washed three times, and resuspended in RPMI 1640 containing 10% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 U/ml) at a concentration of 2.5×10^6/ml. Cell viability was evaluated with the trypan blue exclusion test. At least 90% of the cells thus obtained were monocytes as determined by a Becton Dickinson FACS analyzer (Becton Dickinson, Mountain View, Calif.) with the monoclonal antibody CD 14 (Boehringer Mannheim). Monocytes were suspended in complete medium at 37°C in a humidified atmosphere containing 5% CO_2.

(ii) Endothelial cell isolation. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins according to the method of Gimbrone (13). The umbilical vein was cannulated, washed with phosphate-buffered saline (PBS), perfused for 20 min with collagenase (1 mg/ml) (Boehringer Mannheim) in PBS at 37°C in 5% CO_2, and rinsed with PBS to detach the cells. HUVEC were collected and established as primary cultures in medium 199 (M199) (GIBCO BRL, Grand Island, N.Y.) containing 20% FCS (GIBCO BRL). Cells were serially passaged and maintained by using M199 supplemented with 20% FCS and porcine intestinal heparin (50 μg/ml; Sigma Chemical Co.) in tissue culture flasks precoated with 0.1% gelatin. For experimental use, passages 2 and 3 of HUVEC were plated on 24-well tissue culture plates coated with 0.1% gelatin and grown until reaching confluence.

Up-regulation of adhesion molecules. HUVEC (10^5) were plated in 6-well tissue culture plates coated with 0.1% gelatin and incubated at 37°C overnight. HSPs, bovine serum albumin (BSA) (Sigma Chemical Co.), or *E. coli* lipopolysaccharide (LPS) (Sigma Chemical Co.) was added 4, 6, and 24 h prior to staining the cells for E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1). After incubation at 37°C for different

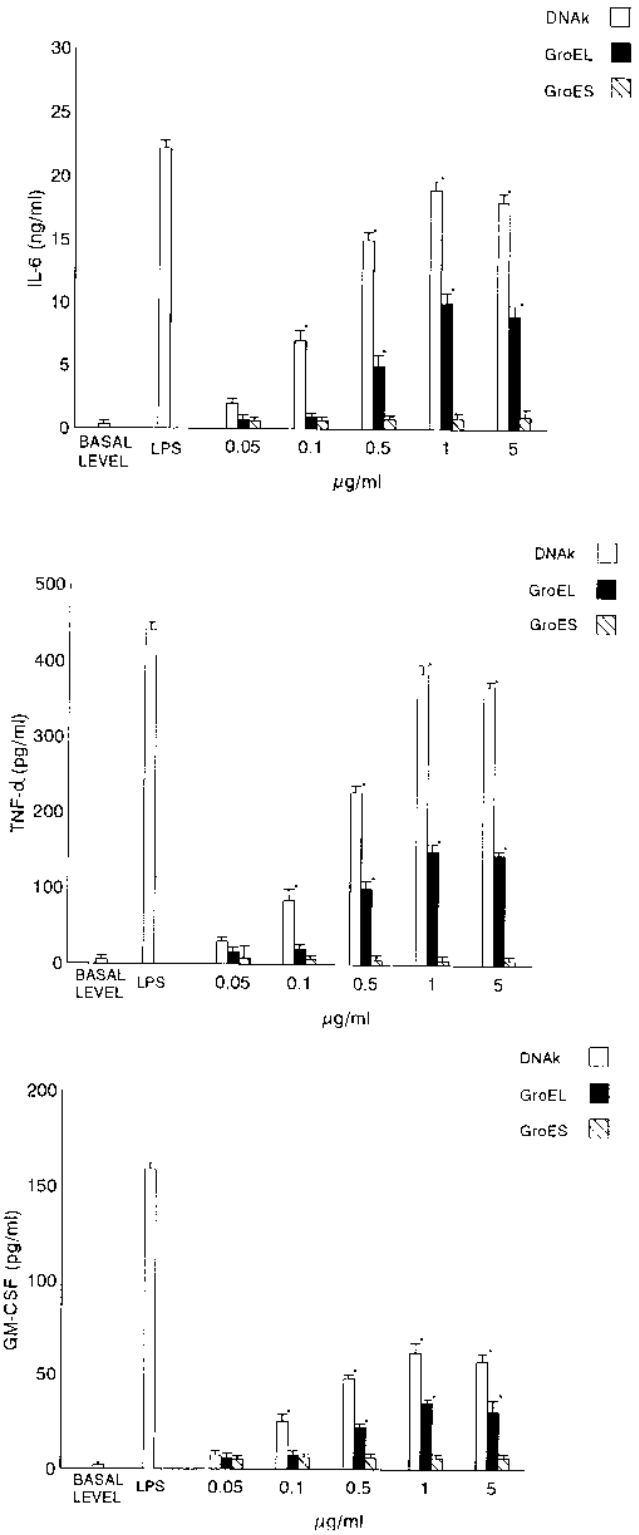

FIG. 2. Release of GM-CSF, IL-6, and TNF-α from human monocytes incubated with LPS (1 μg/ml), BSA (10 μg/ml), GroES (0.05 to 5 μg/ml) (5×10^{-3} to 5×10^{-1} nmol/ml), GroEL (0.05 to 5 μg/ml) (8.33×10^{-4} to 8.33×10^{-2} nmol/ml), or DNAk (0.05 to 5 μg/ml) (7.14×10^{-4} to 7.14×10^{-2} nmol/ml). Each point represents the mean of three experiments ± standard deviation. Points designated by asterisks indicate statistically significant differences ($P < 0.05$) versus untreated cells.

TABLE 1. Effect of specific MAbs on the release of IL-6, TNF-α, and GM-CSF induced by DNAk and GroEL in human monocytes[a]

Challenge	Amt released		
	IL-6 (ng/ml)	TNF-α (pg/ml)	GM-CSF (pg/ml)
None	0.7 ± 0.4	7.1 ± 2.6	2.7 ± 1.5
GroEL	9.3 ± 3.1	156.2 ± 12.5	45.3 ± 6.7
GroEL + anti-GroEL	0.9 ± 0.5	7.8 ± 2.7	2.9 ± 1.7
DNAk	18.2 ± 4.2	392.3 ± 19.7	45.5 ± 6.8
DNAk + anti-DNAk	0.9 ± 0.4	7.8 ± 2.8	2.9 ± 1.6

[a] Monocytes were treated for 24 h with either culture medium only, HSPs (1 μg/ml), or HSPs pretreated with specific MAb as described in Materials and Methods. Data are expressed as means ± standard deviations of three separate experiments.

lengths of time, HUVEC 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 nonenzymatically harvested HUVEC. HUVEC surface E-selectin, ICAM-1, and VCAM-1 expression was determined by indirect immunofluorescence. HUVEC were stained with a saturating 1:200 concentration of primary monoclonal antibody (MAb) and a saturating 1:20 concentration of secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma Chemical Co.) and quantitated by using a Becton Dickinson FACS analyzer. Primary MAbs used included mouse anti-human E-selectin (Bender MedSystems, Vienna, Austria), mouse anti-human ICAM-1/CD54 (Boehringer Mannheim), mouse anti-human VCAM-1 (Genzyme, Cambridge, Mass.), and a mouse IgG1 isotype control antibody. Usually 5,000 cells per sample were assessed, and data are given as percent E-selectin-, ICAM-1-, and VCAM-1-reactive cells in comparison to unstimulated control cells.

Cytokine and adhesion molecule release. Human monocytes (2.5×10^6 cells/ml) and confluent endothelial monolayers were treated with complete medium containing 0.05, 0.1, 0.5, 1, or 5 μg of HSPs per ml; 10 μg of BSA per ml; or 1 μg of LPS per ml. The incubation times were 24 h for cytokine assay and 48 h for adhesion molecule release. All measurements were done with MAbs. GM-CSF, IL-6, TNF-α, soluble E-selectin, ICAM-1, and VCAM-1 were measured by immunoenzymatic methods (Predicta GM-CSF enzyme-linked immunosorbent assay [ELISA] kit, Predicta TNF-α ELISA kit, Predicta IL-6 ELISA kit, Genzyme; soluble E-selectin ELISA kit, soluble ICAM-1 ELISA kit, and soluble VCAM-1 ELISA kit; British Bio-Technology, Abingdon, United Kingdom).

Lactate dehydrogenase (LDH) assay. The LDH assay was done according to manufacturer's instructions (Boehringer Mannheim).

RNA isolation and cDNA preparation. HUVEC and monocytes (10^7) were collected 3 h after stimulation, and total RNA was extracted according to the method of Chomczynski (3). The RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried, and dissolved in 15 μl of RNase-free water. One microgram of oligo(dT) (Promega Biotec, Madison, Wis.) 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 dithiothreitol (Sigma Chemical Co.); 1 mM (each) dATP, dGTP, dCTP, and dTTP; 35 U of RNasin (Promega); and 525 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) in reverse transcription buffer.

PCR procedure. PCR primers for human GM-CSF, IL-6, and β-actin were purchased from Clontech (Palo Alto, Calif.). Human adhesion molecule primer pairs sequences were designed on the basis of published gene sequences as follows: E-selectin, 3′GCGAGGCTACATGAATTGTCTTC, 5′GAGCTGCAG AGCCATTGAGCG; ICAM-1, 3′CGTGCCGCACTGAACTGGAC, 5′CCTCA CACTTCACTGTCACCT; VCAM-1, 3′TGATGACAGTGTCTCCTTCTTTG, 5′ATCCCTACCATTGAAGATACTGG. The primer sequences were complementary to sequences in the exons or spanned exon-exon junctions and thus were RNA specific.

Two microliters 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 of *Taq* DNA polymerase (Promega) in a final volume of 50 μl of *Taq* DNA polymerase 10× buffer (Promega). The PCR was performed in a Perkin-Elmer thermal cycler for 30 cycles as follows: 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 3 min of extension at 72°C. The reaction product was visualized by electrophoresis using 25 μl of the reaction mixture at 100 V in a 1.5% agarose gel containing ethidium bromide (1 μg/ml). The gels were then examined on a UV light box and photographed. One microgram of *Bgl*I- and *Hin*fI-digested pBR328 DNA (Boehringer Mannheim) was run in parallel to generate molecular weight markers (proving bands at 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, 220, and 154 bp).

Blocking experiments. To verify whether the effects were due to HSP properties, they were inhibited by available specific MAbs before cytokines and adhesion molecule expression and release were assayed. To this aim, cell cultures were stimulated with HSPs pretreated for 1 h at 4°C with MAbs anti-DNAk (clone 8E2) and anti-GroEL (clone 9A1) (Stressgene Biotechnologies Corp., Victoria, Canada) (1:100 dilution). To assess whether the expression of adhesion molecules is a direct or indirect effect due to HSPs, monoclonal antibody anti-human IL-6 and anti-human GM-CSF (Genzyme) were used to block E-selectin, ICAM-1, and VCAM-1 cytokine induction. The antibodies (0.5 μg/ml) were added to cell culture media immediately following HSP stimulation. HUVEC were incubated for the usual length of time before adhesion molecule surface expression and release were assayed. Adhesion molecule mRNA expression was also checked by reverse transcription (RT)-PCR analysis.

Statistics. All experiments were carried out in triplicate; results were expressed as the means ± standard deviations. Comparisons between tests were made by Student's *t* test, with statistical significance considered to be $P < 0.05$.

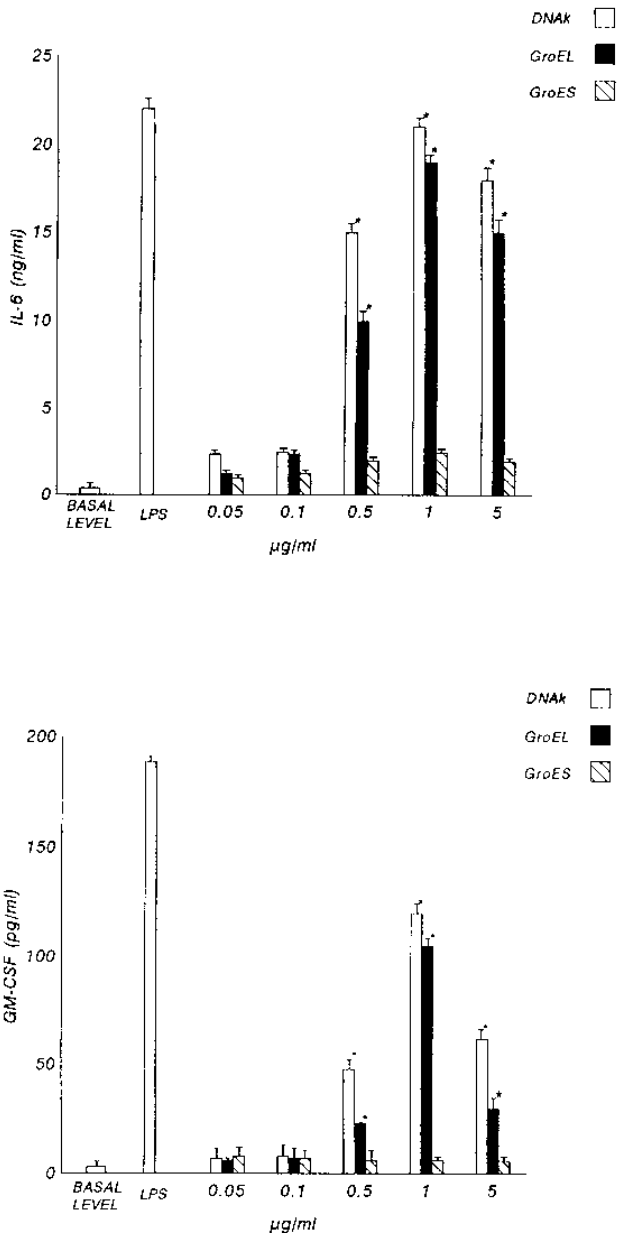

FIG. 3. Release of GM-CSF and IL-6 from HUVEC. Incubation conditions, cytokines, and *P* values are as shown in the legend to Fig. 2.

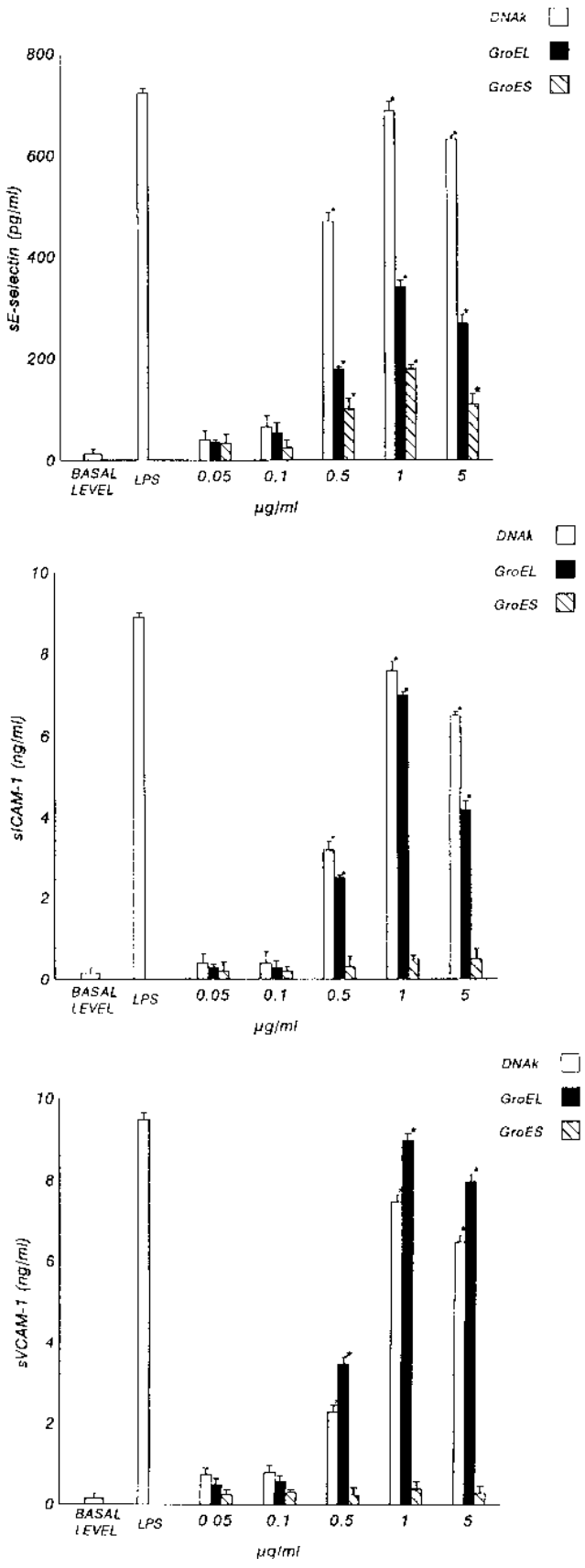

FIG. 4. Release of sE-selectin, sICAM-1, and sVCAM-1 from HUVEC. Incubation conditions, cytokines, and *P* values are as shown in the legend to Fig. 2.

RESULTS

Purity of HSP preparations. The purity of HSP preparations was checked by high-performance liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All possible traces of LPS were identified by gel electrophoresis minislabs stained with silver nitrate as described by Tsai and Frasch (31) and by the *Limulus* amoebocyte lysate assay. As revealed by silver nitrate-stained SDS-PAGE, there were no traces of LPS in HSP preparations (Fig. 1). The *Limulus* amoebocyte lysate test 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 (14 pg/ml) (QCL-1000 Quantitative Chromogenic LAL; Biowhittaker Inc., Walkersville, Md.). All HSPs at the concentrations used were negative on the *Limulus* amoebocyte lysate test.

In addition, to neutralize the biological activity of LPS traces that could be present in the preparation, HSPs were incubated with polymyxin B (Sigma Chemical Co.) at room temperature for 1 h in a ratio of 1:10. In all of the tests performed, HSPs plus polymyxin B gave the same results as HSPs alone.

IL-6, TNF-α, and GM-CSF release by human monocytes stimulated with HSPs. As shown in Fig. 2, DNAk was able to induce a dose-dependent release of these cytokines starting from a concentration of 0.1 μg/ml (1.43×10^{-3} nmol/ml) with a maximum activity at 1 μg/ml (1.43×10^{-2} nmol/ml). IL-6 and TNF-α maximum release was similar to that obtained when cells were stimulated with LPS. Concentrations higher than 1 μg/ml of DNAk (1.43×10^{-2} nmol/ml) had no further effect on the production of IL-6, TNF-α, and GM-CSF, and concentrations lower than 0.1 μg/ml (1.43×10^{-3} nmol/ml) had no significant effect. Stimulation with GroEL induced a lower release of IL-6, TNF-α, and GM-CSF starting at a concentration of 0.5 μg/ml (8.33×10^{-3} nmol/ml) and showed its maximum activity at 1 μg/ml (1.66×10^{-2} nmol/ml). No significant IL-6, TNF-α, and GM-CSF release was detected when cells were treated with GroES even when a concentration of 5 μg/ml (5×10^{-1} nmol/ml) was used (Fig. 2). Cytokine release in response to BSA (10 μg/ml) was not significantly different compared to basal level.

TNF-α release in response to DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) and GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml) began after 12 h of incubation and reached its highest level after 24 h. Time kinetics of IL-6 and GM-CSF release from human monocytes stimulated with DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) and GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml) revealed an earlier activation compared to that observed for TNF-α release showing the maximum level after 24 h of incubation (data not shown). As shown in Table 1, MAbs anti-DNAk and anti-GroEL inhibited the cytokine-inducing activity showed by HSPs in our experimental conditions.

GM-CSF, IL-6, sE-selectin, sICAM-1, and sVCAM-1 release by endothelial cells stimulated with HSPs. The amount of GM-CSF and IL-6 produced by HUVEC monolayers after 24 h of incubation with DNAk and GroEL was significantly higher than that released by HUVEC incubated with BSA (10 μg/ml) or medium only (Fig. 3). This amount increased progressively up to a DNAk concentration of 1 μg/ml (1.43×10^{-2} nmol/ml) and a GroEL concentration of 1 μg/ml (1.66×10^{-2} nmol/ml). GM-CSF release was, however, lower with respect to that obtained by using LPS as a stimulus. On the other hand, IL-6 maximum release was similar to that obtained when cells were stimulated with LPS. DNAk and GroEL concentrations higher than 1 μg/ml (1.43×10^{-2} and 1.66×10^{-2} nmol/ml, respectively) showed no further effect on GM-CSF and IL-6

TABLE 2. Effect of specific MAbs on the release of IL-6, GM-CSF, E-selectin, ICAM-1, and VCAM-1 induced by HSPs in human endothelial cells[a]

Challenge	Amt released				
	IL-6 (ng/ml)	GM-CSF (pg/ml)	E-selectin (pg/ml)	ICAM-1 (ng/ml)	VCAM-1 (ng/ml)
None	0.5 ± 0.2	2.2 ± 1.4	11 ± 3.2	0.13 ± 0.07	0.15 ± 0.07
GroES	2.1 ± 1.4	4.5 ± 2.1	193 ± 14.1	0.52 ± 0.12	0.45 ± 0.23
GroES + anti-IL-6			188 ± 13.6	0.49 ± 0.10	0.41 ± 0.21
GroES + anti-GM-CSF			190 ± 14.5	0.55 ± 0.14	0.48 ± 0.33
GroEL	18.6 ± 4.3	102 ± 10.1	325 ± 18.2	6.77 ± 2.51	9.05 ± 3.12
GroEL + anti-GroEL	0.6 ± 0.2	1.8 ± 1.3	14 ± 3.6	0.15 ± 0.07	0.20 ± 0.11
GroEL + anti-IL-6			327 ± 18.3	6.59 ± 2.41	8.95 ± 2.98
GroEL + anti-GM-CSF			322 ± 17.4	6.70 ± 2.62	9.01 ± 3.01
DNAk	21.7 ± 4.6	120 ± 10.9	685 ± 26.2	6.95 ± 2.71	7.52 ± 2.74
DNAk + anti-DNAk	0.7 ± 0.3	2.5 ± 1.6	13 ± 3.7	0.16 ± 0.08	0.18 ± 0.09
DNAk + anti-IL-6			678 ± 25.6	6.86 ± 2.63	7.48 ± 2.71
DNAk + anti-GM-CSF			687 ± 27.4	6.79 ± 2.51	7.55 ± 2.73

[a] HUVEC were treated for up to 48 h with either culture medium only, HSPs (1 μg/ml), or HSPs in the presence of MAb anti-IL-6 or GM-CSF. Cells were also stimulated with GroEL pretreated with MAb anti-GroEL and DNAk pretreated with MAb anti-DNAk as described in Materials and Methods. Data are expressed as means ± standard deviations of three separate experiments.

secretion, and concentrations lower than 0.5 μg/ml (7.14×10^{-3} and 8.33×10^{-3} nmol/ml, respectively) had no significant effect.

The dose-dependent release of E-selectin, sICAM-1, and sVCAM-1 is shown in Fig. 4. HUVEC stimulated for 48 h with *E. coli* DNAk, GroEL, and GroES showed a dose-dependent release of sE-selectin starting from a 0.5-μg/ml concentration (7.14×10^{-3}, 8.33×10^{-3}, and 5×10^{-2} nmol/ml, respectively), with a maximum activity at a concentration of 1 μg/ml (1.43×10^{-2}, 1.66×10^{-2}, and 1×10^{-1} nmol/ml, respectively). When cells were treated with DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml), sE-selectin release was similar to that observed when cells were incubated with LPS. GroEL and GroES were also able to induce sE-selectin release but at a lower rate.

Among HSPs, only DNAk and GroEL induced a significant release of sICAM-1 and sVCAM-1 starting from a 0.5-μg/ml (7.14×10^{-3} and 8.33×10^{-3} nmol/ml, respectively) concentration, with a maximum activity at 1 μg/ml (1.43×10^{-2} and 1.66×10^{-2} nmol/ml, respectively). Their effect on sICAM-1 and sVCAM-1 release was similar to that shown by LPS. GroES was not able to induce significant GM-CSF, IL-6, sICAM-1, and sVCAM-1 release. BSA (10 μg/ml) induced an adhesion molecule release similar to basal level.

The GM-CSF and IL-6 release in response to DNAk and GroEL never occurred before 12 h after exposure and was complete after 24 h. DNAk, GroEL, and GroES began to induce a significant sE-selectin release after 24 h of incubation, reaching the maximum level after 48 h. sICAM-1 and sVCAM-1 release showed an earlier activation, reaching a significant level already after 12 h of stimulation (data not shown). As shown in Table 2, DNAk and GroEL pretreated with MAbs anti-DNAk and anti-GroEL did not show any biological activity. Furthermore MAbs to GM-CSF did not sig-

TABLE 3. E-selectin surface expression after treatment with *E. coli* HSPs[a]

Challenge	Amt expressed (pg/ml) at time (h)		
	4	6	24
None	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.1
BSA	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.3
LPS	6.2 ± 2.3	9.8 ± 3.1	0.8 ± 0.6
GroES	2.3 ± 1.4	13.3 ± 3.6	0.5 ± 0.3
GroES + anti-IL-6	2.5 ± 1.7	13.0 ± 3.7	0.6 ± 0.4
GroES + anti-GM-CSF	2.1 ± 1.4	12.9 ± 3.5	0.4 ± 0.1
GroEL	3.4 ± 1.8	17.3 ± 4.1	0.7 ± 0.3
GroEL + anti-GroEL	0.3 ± 0.2	0.4 ± 0.3	0.4 ± 0.1
GroEL + anti-IL-6	3.2 ± 1.7	16.9 ± 4.1	0.6 ± 0.3
GroEL + anti-GM-CSF	3.6 ± 1.9	17.1 ± 4.2	0.8 ± 0.4
DNAk	5.5 ± 2.3	20.2 ± 4.6	0.8 ± 0.5
DNAk + anti-DNAk	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.2
DNAk + anti-IL-6	5.2 ± 2.2	19.7 ± 4.4	0.7 ± 0.3
DNAk + anti-GM-CSF	5.0 ± 2.1	20.1 ± 4.4	0.9 ± 0.5

[a] HUVEC were treated for 4, 6, and 24 h with either culture medium only, BSA (10 μg/ml), *E. coli* LPS (1 μg/ml), HSPs (1 μg/ml), or HSPs in the presence of MAb anti-IL-6 or GM-CSF. Cells were also stimulated with GroEL pretreated with MAb anti-GroEL and DNAk pretreated with MAb anti-DNAk. E-selectin surface expression was assessed cytofluorometrically as described in Materials and Methods. IgG1 isotype controls were done for each cell population tested and did not exceed background fluorescence. Data are given as means ± standard deviations of three separate experiments.

TABLE 4. ICAM-1 surface expression after treatment with *E. coli* HSPs[a]

Challenge	Amt expressed (pg/ml) at time (h)		
	4	6	24
None	2.3 ± 1.2	3.4 ± 1.5	2.5 ± 1.5
BSA	2.8 ± 1.5	4.1 ± 2.1	3.2 ± 1.7
LPS	9.5 ± 3.2	12.8 ± 3.5	22.4 ± 4.6
GroES	2.8 ± 1.5	2.3 ± 1.4	2.9 ± 1.7
GroES + anti-IL-6	2.5 ± 1.4	2.8 ± 1.4	3.0 ± 1.7
GroES + anti-GM-CSF	2.9 ± 1.5	3.0 ± 1.7	3.2 ± 1.8
GroEL	8.6 ± 2.8	11.9 ± 3.4	20.4 ± 4.5
GroEL + anti-GroEL	2.1 ± 1.3	2.9 ± 1.7	2.4 ± 1.2
GroEL + anti-IL-6	8.2 ± 2.7	11.5 ± 3.3	19.6 ± 4.4
GroEL + anti-GM-CSF	9.0 ± 2.9	12.2 ± 3.4	19.8 ± 4.3
DNAk	7.3 ± 2.7	12.8 ± 3.5	21.5 ± 4.6
DNAk + anti-DNAk	2.1 ± 0.8	2.9 ± 1.4	2.4 ± 1.3
DNAk + anti-IL-6	7.6 ± 2.7	12.1 ± 3.3	20.4 ± 4.5
DNAk + anti-GM-CSF	7.0 ± 2.5	13.1 ± 3.6	21.4 ± 4.6

[a] HUVEC were treated as described in Table 3, footnote *a*. ICAM-1 surface expression was assessed cytofluorometrically as described in Materials and Methods. IgG1 isotype controls were done for each cell population tested and did not exceed background fluorescence. Data are given as means ± standard deviations of three separate experiments.

TABLE 5. VCAM-1 surface expression after treatment with *E. coli* HSPs[a]

Challenge	Amt expressed (ng/ml) at time (h)		
	4	6	24
None	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
BSA	0.4 ± 0.2	0.5 ± 0.3	0.5 ± 0.2
LPS	17.1 ± 4.1	19.3 ± 4.4	16.9 ± 3.9
GroES	0.5 ± 0.3	0.4 ± 0.2	0.4 ± 0.1
GroES + anti-IL-6	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.2
GroES + anti-GM-CSF	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1
GroEL	14.5 ± 3.8	16.4 ± 4.1	15.3 ± 3.9
GroEL + anti-GroEL	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.2
GroEL + anti-IL-6	14.9 ± 3.7	16.8 ± 4.0	15.1 ± 3.7
GroEL + anti-GM-CSF	14.1 ± 3.6	16.0 ± 3.9	15.5 ± 3.9
DNAk	16.8 ± 4.1	18.2 ± 4.2	16.3 ± 3.8
DNAk + anti-DNAk	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.3
DNAk + anti-IL-6	17.0 ± 4.2	18.8 ± 4.3	16.7 ± 4.8
DNAk + anti-GM-CSF	16.6 ± 3.9	17.9 ± 4.2	15.9 ± 3.8

[a] HUVEC were treated as described in Table 3, footnote *a*. VCAM-1 surface expression was assessed cytofluorometrically as described in Materials and Methods. IgG1 isotype controls were done for each cell population tested and did not exceed background fluorescence. Data are given as means ± standard deviations of three separate experiments.

nificantly modify adhesion molecule release in HUVEC stimulated with HSPs.

Bacterial HSPs up-regulate E-selectin, ICAM-1, and VCAM-1 expression on HUVEC surface. As shown in Table 3, E-selectin was not expressed at a basal level on the surface of HUVEC but could be induced by treatment with LPS (1 μg/ml), DNAk, GroEL, and GroES (1 μg/ml) (1.43×10^{-2}, 1.66×10^{-2}, and 1×10^{-1} nmol/ml, respectively). These effects began after 4 h of stimulation and reached a peak at 6 h. After 24 h, E-selectin surface expression returned to the basal level.

As detected by flow cytometry, HUVEC expressed ICAM-1 basally but can be induced to express higher levels after stimulation with LPS (1 μg/ml), DNAk, and GroEL (1 μg/ml) (1.43×10^{-2} and 1.66×10^{-2} nmol/ml, respectively). The increase of ICAM-1 levels began to appear within 4 h of stimulation, and this up-regulation continued up to 24 h (Table 4). VCAM-1 was not expressed basally on the HUVEC surface but could be induced by stimulation with LPS (1 μg/ml), DNAk, and GroEL (1 μg/ml) (1.43×10^{-2} and 1.66×10^{-2} nmol/ml, respectively). As shown in Table 5, these effects began after 4 h of incubation and reached a peak after 6 h. The number of VCAM-1-positive cells remained relatively unchanged at 24 h after stimulation. BSA (10 μg/ml) did not induce an enhanced surface expression of E-selectin, ICAM-1, and VCAM-1. As assessed by flow cytometry, MAbs anti-DNAk and anti-GroEL inhibited the increase of adhesion molecule surface expression induced by HSPs (Tables 3 to 5). This increase was a direct effect because the presence of MAbs anti-IL-6 and anti-GM-CSF used to block adhesion cytokine induction did not significantly modify the expression of E-selectin, ICAM-1, and VCAM-1 in HUVEC stimulated with HSPs (Tables 3 to 5).

IL-6, TNF-α, and GM-CSF mRNA expression in human monocytes stimulated with HSPs. Figure 5 shows a representative gel of the cytokine RT-PCR products of RNA extracted from HSP-treated monocytes. Cells were stimulated with HSP concentrations that showed the best activity in inducing the release of IL-6, TNF-α, and GM-CSF.

The intensity of the signal from IL-6, TNF-α, and GM-CSF mRNA increased when cells were treated with DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) and with GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml) but at a lower rate. No effect on the level of cytokine mRNA was detected when cells were treated with GroES. BSA did not modify the expression of cytokine mRNAs compared to cells incubated in medium only (Fig. 5). DNAk and GroEL pretreated with MAbs anti-DNAk and anti-GroEL were not able to induce any increase in cytokine mRNA expression (Fig. 5).

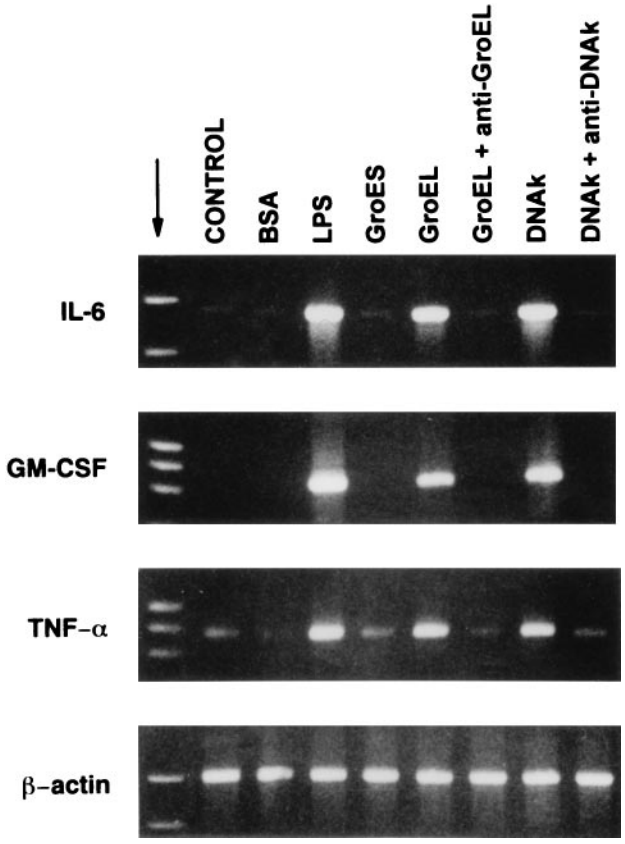

FIG. 5. Effect of HSPs on the steady-state levels of GM-CSF, IL-6, and TNF-α mRNA in human monocytes. Cultures were treated for 3 h with culture medium (control), BSA (10 μg/ml), *E. coli* LPS (1 μg/ml), GroES (1 μg/ml) (1×10^{-1} nmol/ml), GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml), GroEL (1 μg/ml) pretreated with MAb anti-GroEL, DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml), or DNAk (1 μg/ml) pretreated with MAb anti-DNAk and then subjected to RNA extraction and RT-PCR amplification. Reaction products were run on a 1.5% agarose gel in the presence of appropriate molecular weight markers (arrow); β-actin was the positive transcription control.

GM-CSF, IL-6, and adhesion molecule mRNA expression in HUVEC stimulated with HSPs. Cells were stimulated with HSP concentrations that showed the best activity in inducing the release of IL-6, GM-CSF, and adhesion molecules.

The effect of *E. coli* HSPs on GM-CSF and IL-6 mRNA expression in HUVEC is shown in Fig. 6. Stimulation with DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) greatly increased the cellular mRNA levels of GM-CSF; however, stimulation with GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml) and GroES (0.05 to 5 μg/ml) (5×10^{-3} to 5×10^{-1} nmol/ml) induced either little or no mRNA expression. IL-6 mRNA expression was also increased by GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml) and DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) treatment. Stimulation with GroES (0.05 to 5 μg/ml) (5×10^{-3} to 5×10^{-1} nmol/ml) induced no IL-6 mRNA.

The effect of HSP on E-selectin, ICAM-1, and VCAM-1

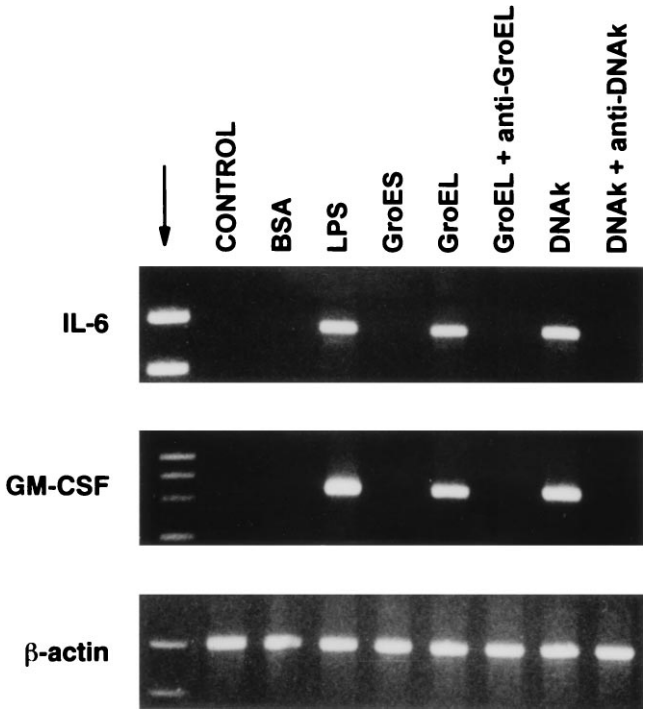

FIG. 6. Effect of HSPs on the steady-state levels of GM-CSF and IL-6 mRNA in human endothelial cells. Culture conditions were as described in the legend to Fig. 5.

mRNA expression in human endothelial cells is shown in Fig. 7. Stimulation with DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) greatly increased the cellular mRNA levels of E-selectin. Treatment with GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml) and GroES (1 μg/ml) (1×10^{-1} nmol/ml) induced little E-selectin mRNA expression. The intensity of the signal from ICAM-1 mRNA increased when cells were treated with DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) and GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml). VCAM-1 mRNA expression increased when cells were treated with DNAk (1 μg/ml) (1.43×10^{-2} nmol/ml) and GroEL (1 μg/ml) (1.66×10^{-2} nmol/ml). However, stimulation with DNAk induced a lower expression of VCAM-1 mRNA compared to GroEL. No effect on the level of ICAM-1 and VCAM-1 mRNA was detected when cells were treated with GroES at concentrations ranging from 0.05 to 5 μg/ml (5×10^{-3} to 5×10^{-1} nmol/ml). BSA did not modify the expression of cytokine and adhesion molecule mRNAs compared to cells incubated in medium only (Fig. 6 and 7). DNAk and GroEL pretreated with MAbs anti-DNAk and anti-GroEL did not show any biological activity in our experimental conditions. Furthermore, MAbs anti-IL-6 and anti-GM-CSF did not significantly modify adhesion molecule expression in HU VEC stimulated with HSPs.

DISCUSSION

The role played by HSPs during the host response has not been yet clarified. It is well-known that a variety of stressful

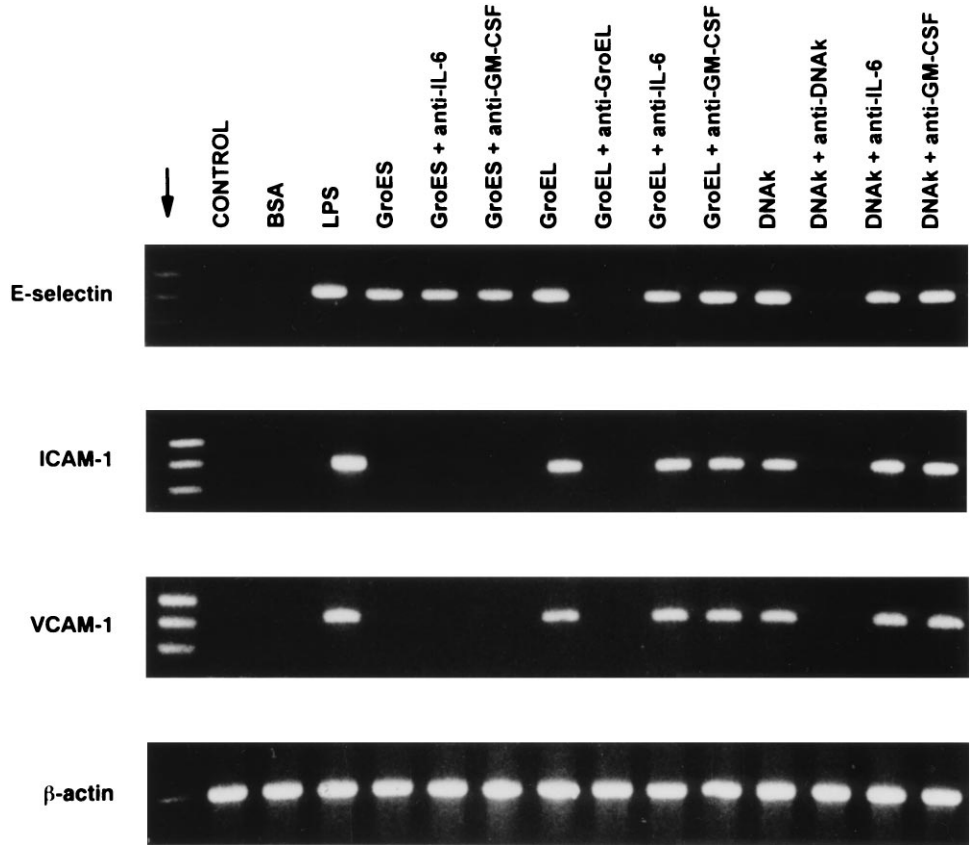

FIG. 7. Effect of HSPs on the steady-state levels of E-selectin, ICAM-1, and VCAM-1 mRNA in human endothelial cells. Culture conditions were as described in the legend to Fig. 5. Cells were also stimulated with HSPs in the presence of MAb anti-IL-6 and anti-GM-CSF. Cell cultures were then subjected to RNA extraction and RT-PCR amplification. Reaction products were run on 1.5% agarose gel in the presence of appropriate molecular weight markers (arrow); β-actin was the positive transcription control.

stimuli, including heat shock, nutrient deprivation, oxygen radicals (H_2O_2), and cytokines, induce a marked increase in HSP synthesis and production (8, 14, 21). Therefore, it is likely that, at the inflammatory site, the 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 nonspecific responses affecting their functions (16, 17, 34).

Our study demonstrated that among HSPs, DNAk and GroEL were able to induce the release of GM-CSF and IL-6 from HUVEC. HSP treatment of monocytes also affects, in a dose-dependent manner, GM-CSF, TNF-α, and IL-6 release. DNAk is able to induce TNF-α and IL-6 release similar to that observed when cells were stimulated with LPS, while a lower activity was shown by GroEL at the same concentrations. No significant effect was elicited when cells were stimulated with GroES. Among HSPs, DNAk and GroEL were able to induce the release of soluble forms of E-selectin, ICAM-1, and VCAM-1, while GroES showed a significant activity only on E-selectin release.

Release of cell surface adhesins may simply be a mechanism for breaking adhesive interactions between cells or may provide a means for clearing the cell surface of adhesins to control adhesivity. The soluble forms may themselves have biological functions. Recombinant soluble adhesins can partially block adhesion of leukocytes to surface-bound adhesins; soluble forms at locally high concentrations may therefore also act to inhibit adhesive interactions. Binding of soluble adhesins to their target cells may also deliver an activating stimulus; recombinant soluble E-selectin has been shown to be a neutrophil chemoattractant and to activate the integrin CR3 (11, 23).

Cytokine and adhesion molecule release was not due to cell lysis; LDH, as a cytoplasmic marker, was found to be present in the supernatant of activated cells at a level similar to that detected in the supernatant of nonactivated cells (data not shown).

Our study also demonstrated that among HSPs, DNAk and GroEL were able to increase the surface expression of E-selectin, ICAM-1, and VCAM-1 in HUVEC. GroES induces an increase only of E-selectin surface expression.

All these findings were confirmed by RT-PCR analysis of total RNA extracted from HUVEC and monocytes treated with the HSPs.

Adhesion molecule expression and release seem to be a direct effect of HSPs and not via cytokines because MAbs anti-human IL-6 and anti-human GM-CSF used to block E-selectin, ICAM-1, and VCAM-1 cytokine induction did not modify the adhesin expression and release induced by HSP treatment.

The main conclusions of this study are that DNAk and GroEL by themselves induce activation of human monocytes and endothelial cells as demonstrated by their ability to express and release cytokines and adhesion molecules. These effects are due to HSP properties because they are inhibited by specific MAbs. Furthermore, incubation of cells with BSA, which served as control protein, does not lead to cell activation.

The effects of HSPs cannot be due to LPS contamination. The amount of LPS needed to induce similar biological activities is much larger than that present in our preparations (10). Furthermore, control experiments done with polymyxin B to neutralize LPS traces that could be present in HSP preparations gave the same results as those with HSPs used alone.

The mechanisms involved in cell activation have not yet been clarified. It is well-known that HSPs, in their role of molecular chaperones, have a high affinity for immature proteins, binding them during the protein folding and unfolding process. Therefore it is likely that HSPs are able to bind cell surface receptors providing the signal required for cell activation. Monocyte and endothelial cell activation could also be due to aspecific perturbations within the lipoproteic phase of the cytoplasmic membrane.

Among *E. coli* HSPs, DNAk shows a higher activity with respect to GroEL. The reason for the relatively lower potency of GroES in comparison with the other HSPs, even if used at the same molarity, is not clear. It might be related to the relatively small size of this protein, which prohibits it from effectively cross-linking receptors following binding.

Our findings provide further evidence for the hypothesis that HSPs may play an important role in the initiation of the inflammatory process that accompanies infections with microbial pathogens by regulating the expression of cytokines involved in the activation of leukocytes and endothelial cells.

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