

## In Vivo Production of Heat Shock Protein in Mouse Peritoneal Macrophages by Administration of Lipopolysaccharide

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**The in vivo production of heat shock protein was studied by administration of bacterial lipopolysaccharide (LPS) into mice. Heat shock protein 70 was detected in the extract of adherent peritoneal cells from mice injected intraperitoneally with LPS by using the immunoblotting method. The expression of heat shock protein 70 was found 2 days after injection of LPS and reached its peak 4 days after injection. The intraperitoneal injection of LPS induced the expression of heat shock protein 70, whereas its subcutaneous injection did not. The in vivo production of heat shock protein 70 was inhibited by administration of LPS together with quercetin, an inhibitor of accumulation of heat shock protein 70 mRNA. Tumor necrosis factor alpha enhanced LPS-induced heat shock protein production in vivo. There was a decrease of  $\gamma\delta$  T cells in the peritoneal cavity of mice injected intraperitoneally with LPS. It was suggested that bacterial LPS is a stressful agent which induces the in vivo heat shock protein response, and its administration leads to the production of heat shock protein 70 in peritoneal macrophages.**

Heat shock proteins (HSP) or stress proteins are produced by a broad spectrum of pathogens and have been shown to be among the dominant target antigens recognized in immune responses to pathogens (6, 16, 24, 25). The stress exerted by phagocytes may induce increased HSP synthesis in microbial pathogens in an attempt to protect themselves from the host effector mechanism (1, 6, 11). On the other hand, host cells also need to protect themselves from the noxious molecules which pathogens produce (6). This may be achieved in part by HSP synthesis in host cells. In fact, it has been reported that host cells express increased HSP levels after in vitro infection with a variety of viral pathogens (3, 9, 13). However, less is known about the in vivo HSP response in the host infected with microbial pathogens, although HSP are almost certainly involved in protecting cells from their deleterious effects (24). Lipopolysaccharide (LPS), one of the noxious products of gram-negative organisms, may certainly alter the metabolism of various cells in the host, and host cells may exhibit the HSP response to LPS with similar behavior. However, there are no reports of LPS stimulation of in vivo HSP production by the host.

The HSP70 family is probably one of the best-studied stress proteins found in all prokaryotes and eukaryotes, and it is, in particular, the most highly conserved of HSP families. The highly inducible mammalian HSP70 is hardly detectable under normal conditions but becomes one of the most actively synthesized proteins in cells in response to stress (19, 24). In the present study, we investigated whether and how the administration of LPS to mice caused in vivo induction of the HSP70 family in the host. Here we describe the in vivo production of HSP70 in peritoneal macrophages from mice injected intraperitoneally (i.p.) with LPS.

### MATERIALS AND METHODS

**Animals.** Inbred SMA mice, approximately 6 to 8 weeks of age, were supplied by the Institute for Laboratory Animal Research at Aichi Medical University.

**Reagents.** Anti-HSP70 monoclonal antibodies (SPA-810 and SPA-820) were purchased from StressGen Biotechnologies Corp., Victoria, British Columbia, Canada. Hamster anti-mouse  $\gamma\delta$  T-cell receptor monoclonal antibody (GL3) conjugated with fluorescein isothiocyanate was purchased from Pharmingen Corp., San Diego, Calif. Recombinant mouse gamma interferon (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and interleukin 1 $\alpha$  (IL-1 $\alpha$ ) were purchased from Genzyme Corp., Cambridge, Mass. Quercetin was obtained from Wako Pure Chemical, Osaka, Japan.

**LPS.** The LPS preparation extracted by the phenol-water method from *Escherichia coli* O111:B4 or *Salmonella enteritidis* was obtained from Difco Laboratories, Detroit, Mich. LPS from *Klebsiella pneumoniae* O3 was also prepared by the phenol-water method (20, 22). All experiments were carried out by i.p. administration of LPS from *E. coli* O111 (100  $\mu$ g) unless stated otherwise. Separation of the free lipid A and polysaccharide fraction from *K. pneumoniae* O3 LPS was performed by heating at 100°C for 1 h in 1% acetic acid as described previously (5). Rough-type LPS was prepared from *K. pneumoniae* LEN-113 (O3<sup>-</sup>:K1<sup>-</sup>) (14).

**Preparation of peritoneal cells and adherent cells.** Peritoneal cells were obtained by washing out the peritoneal cavity with RPMI 1640 medium. Two mice per experimental group were used. Separation of nonadherent cells and adherent cells was performed by incubating peritoneal cells in plastic petri dishes for 1 h at 37°C with RPMI 1640 medium containing 5% fetal calf serum, 100 U of penicillin per ml, and 100  $\mu$ g of streptomycin per ml.

**Immunoblot analysis.** Cells were obtained 4 days after injection of LPS unless stated otherwise and washed three times in 0.01 M phosphate-buffered saline at pH 7.2 (PBS). Cell pellets were suspended at a concentration of  $2 \times 10^7$ /ml in a lysis buffer containing 0.5% Nonidet P-40, 0.15 M NaCl, 0.05 M Tris, and 5 mM EDTA at pH 8.0 for 30 min at 4°C. The insoluble debris was removed by microcentrifugation at 18,600

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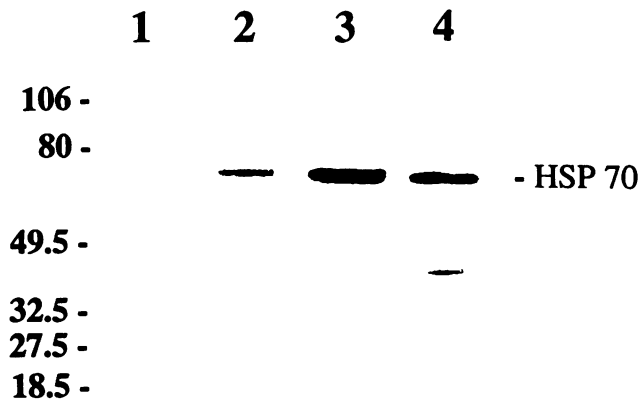


FIG. 1. Time course of the expression of HSP70 in mice injected i.p. with LPS. Peritoneal cells were collected 2, 4, or 9 days after injection of LPS (100  $\mu$ g). The expression of HSP70 was estimated by the immunoblotting method. Lanes: 1, untreated control; 2, 2 days; 3, 4 days; 4, 9 days. Molecular size markers in kilodaltons are on the left.

$\times$  g for 10 min at 4°C. Cell lysates were diluted with an equal volume of sample buffer containing 0.5 M Tris, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol, and 0.05% bromophenol blue and boiled for 2 min. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5 to 20% gradient gel. Proteins separated by SDS-PAGE were transferred to a membrane filter (Durapore; Nihon Millipore Ltd., Tokyo, Japan) by electroblotting. The filters were blocked with 5% skim milk in PBS. After the blots were washed in PBS containing 0.05% Tween 20 (PBS-T), they were treated with a 1:1,000 dilution of anti-HSP70 monoclonal antibody and then washed with PBS-T three times. Resulting immune complexes were reacted with a 1:2,000 dilution of horseradish peroxidase-conjugated protein A (Nippon Bio-Rad Laboratories, Tokyo, Japan) in PBS-T. Finally, labeled antigen bands were detected by an ECL Western blotting (immunoblotting) detection reagent (Amersham, London, United Kingdom). A prestained low-molecular-weight standard kit from Nippon Bio-Rad Laboratories was used as a reference.

**Flow cytometric analysis.** Mice were injected i.p. with LPS (100  $\mu$ g), and peritoneal cells were collected 3 or 5 days after injection. Peritoneal cells were stained with fluorescein isothiocyanate-conjugated anti-mouse  $\gamma\delta$  T-cell receptor antibody (GL3) by a direct immunofluorescence staining method. The stained cells were fixed with 1% paraformaldehyde and analyzed by a FACS-400 flow cytometer (Becton Dickinson, Mountain View, Calif.). The intensity of fluorescence was expressed in a log scale from 1 to  $10^4$ .

## RESULTS

**Detection of HSP70 on peritoneal cells from mice injected i.p. with LPS.** The peritoneal cells, thymocytes, and spleen cells were collected from mice various days after i.p. injection of LPS from *E. coli* O111 (100  $\mu$ g), and the induction of HSP70 was studied by the immunoblotting method. As shown in Fig. 1, HSP70 was found in the extract of peritoneal cells 2 days after injection of LPS. The expression of HSP70 increased and reached its peak 4 days after injection. The high expression of HSP70 lasted longer than 9 days. The qualitative and quantitative time course in the induction of HSP70 was essentially reproducible in every experiment. On the other hand, there

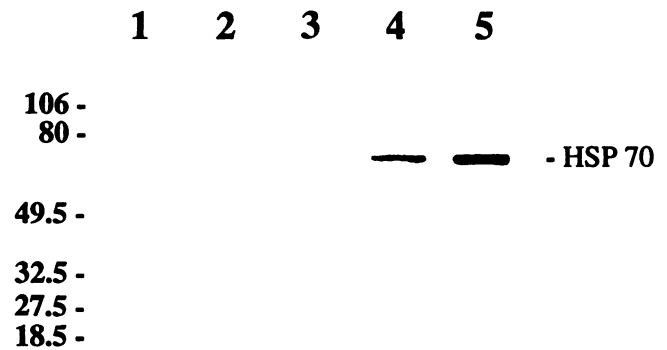


FIG. 2. Dose dependency of the expression of HSP70 on injected LPS. Peritoneal cells were collected 4 days after i.p. injection of various doses of LPS. The expression of HSP70 was estimated by the immunoblotting method. Lanes: 1, untreated control; 2, 1  $\mu$ g; 3, 10  $\mu$ g; 4, 50  $\mu$ g; 5, 100  $\mu$ g. Molecular size markers in kilodaltons are on the left.

was no significant induction of HSP70 in either thymocytes or spleen cells from mice injected with LPS (data not shown).

**Induction of HSP70 on peritoneal cells from mice injected i.p. with various doses of LPS.** The dose dependency of injected LPS on the expression of HSP70 in peritoneal cells was studied. The experimental results are shown in Fig. 2. The level of HSP70 expression was roughly dependent on the dose of LPS injected. The highest expression of HSP70 was found with injection of 100  $\mu$ g of LPS, whereas the injection of 1 or 10  $\mu$ g of LPS did not cause in vivo production of HSP70.

**Comparison of HSP70 induction in peritoneal cells by i.p. and subcutaneous routes of LPS injection.** The inductions of HSP70 in peritoneal cells by i.p. and subcutaneous routes of LPS injection were compared. The experimental results are shown in Fig. 3. The i.p. injection of LPS definitely induced the expression of HSP70, while subcutaneous LPS injection did not induce it in peritoneal cells.

**Expression of HSP70 on adherent peritoneal cells from mice injected i.p. with LPS.** Which cell type in peritoneal cells produced HSP70 by i.p. administration of LPS was studied. Adherent and nonadherent cell fractions were separated by incubating peritoneal cells in plastic dishes. The experimental results are shown in Fig. 4. Injection of LPS induced produc-

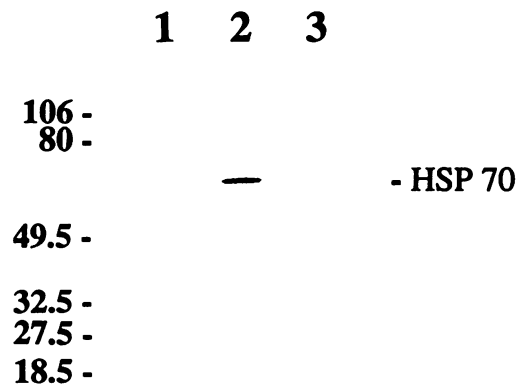


FIG. 3. Comparison of HSP70 production in peritoneal cells induced by i.p. or subcutaneous injection of LPS. Peritoneal cells were collected 4 days after injection of LPS (100  $\mu$ g). The expression of HSP70 was estimated by the immunoblotting method. Lanes: 1, subcutaneous route; 2, i.p. route; 3, untreated control. Molecular size markers in kilodaltons are on the left.

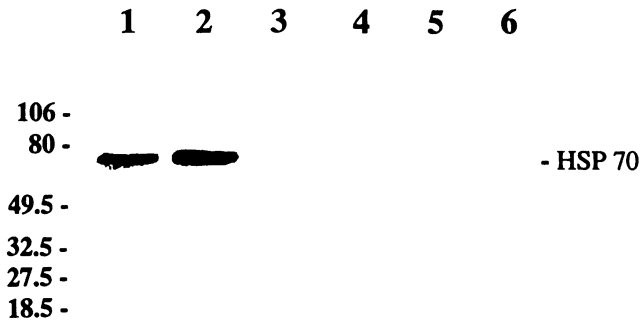


FIG. 4. Expression of HSP70 on adherent peritoneal cells from mice injected i.p. with LPS. Peritoneal cells were collected 4 or 8 days after injection of LPS (100  $\mu$ g). Adherent and nonadherent cells were separated by incubating peritoneal cells in plastic dishes. The expression of HSP70 was estimated by the immunoblotting method. Lanes: 1 to 3, adherent peritoneal cells; 4 to 6, nonadherent peritoneal cells; 1 and 4, 8 days; 2 and 5, 4 days; 3 and 6, untreated control. Molecular size markers in kilodaltons are on the left.

tion of HSP70 only in adherent peritoneal cells, and the expression of HSP70 continued at high levels from 4 to 8 days after injection. On the other hand, there was no significant expression of HSP70 in nonadherent peritoneal cells 4 to 8 days after injection of LPS.

**Inhibitory effect of quercetin on induction of HSP70 in peritoneal cells by administration of LPS.** Quercetin is known to inhibit the induction of HSP, especially the accumulation of HSP70 mRNA (4). To ascertain the HSP70 expression, whether quercetin inhibited the production of HSP70 on peritoneal cells by LPS was tested. LPS with or without quercetin was injected i.p. into mice (50  $\mu$ g). As shown in Fig. 5, the induction of HSP70 by LPS in peritoneal cells was significantly blocked 4 days after injection with quercetin.

**Effects of various cytokines on induction of HSP70 in peritoneal cells by administration of LPS.** Whether a variety of cytokines affected the induction of HSP70 in peritoneal cells by in vivo administration of LPS was determined. IFN- $\gamma$ , TNF- $\alpha$ , or IL-1 $\alpha$  (100 U each) was injected i.p. into mice together with LPS. A typical experimental result is shown in Fig. 6. There

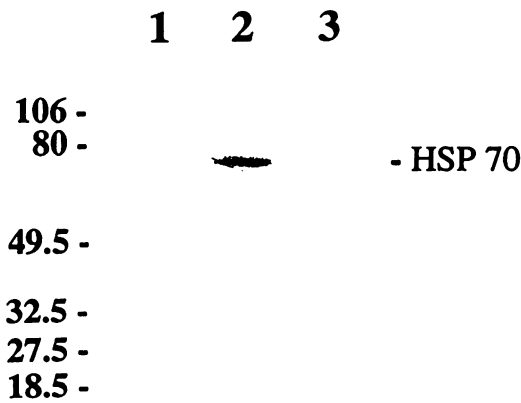


FIG. 5. Inhibitory effect of quercetin on induction of HSP70 in peritoneal cells by administration of LPS. Mice were injected i.p. with LPS (100  $\mu$ g) alone or together with quercetin. Peritoneal cells were collected 4 days after i.p. injection of LPS (100  $\mu$ g). Lanes: 1, untreated control; 2, LPS alone; 3, LPS and quercetin. Molecular size markers in kilodaltons are on the left.

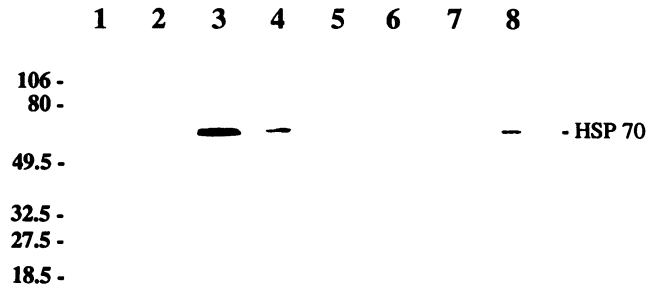


FIG. 6. Effect of various cytokines on induction of HSP70 in peritoneal cells by administration of LPS. Mice were injected i.p. with IFN- $\gamma$ , TNF- $\alpha$ , or IL-1 $\alpha$  (100 U each) in the presence or absence of LPS (100  $\mu$ g). Peritoneal cells were collected 4 days after injection. Lanes: 1, untreated control; 2, LPS and IL-1 $\alpha$ ; 3, LPS and TNF- $\alpha$ ; 4, LPS and IFN- $\gamma$ ; 5, IL-1 $\alpha$ ; 6, TNF- $\alpha$ ; 7, IFN- $\gamma$ ; 8, LPS alone. Molecular size markers in kilodaltons are on the left.

was a marked increase in induction of HSP70 by administration of TNF- $\alpha$  together with LPS. The administration of IFN- $\gamma$  together with LPS slightly enhanced the expression of HSP70, compared with that of LPS alone. The injection of IL-1 $\alpha$  plus LPS inhibited the induction of HSP70 (Fig. 1). In some cases, however, IL-1 $\alpha$  did not show such inhibition. In addition, HSP70 was not induced by in vivo administration of IFN- $\gamma$ , TNF- $\alpha$ , or IL-1 $\alpha$  (100 U each) alone.

**Induction of HSP70 in peritoneal cells by administration of various preparations of LPS.** A comparison of the abilities of various preparations of LPS (100  $\mu$ g) to induce HSP70 in peritoneal cells was done. Administration of LPS from *K. pneumoniae* O3 or *S. enteritidis* could induce expression of HSP70 as well as LPS from *E. coli* could (Fig. 7). Strong expression of HSP70 was found in the case of i.p. injection of rough-type LPS from *K. pneumoniae* O3. The injection of free lipid A from *K. pneumoniae* O3 did not cause the production of HSP70.

**Disappearance of  $\gamma\delta$  T cells from the peritoneal cavity of mice injected i.p. with LPS.** It has been reported that  $\gamma\delta$  T cells respond to HSP and have a particular preference for it (6–8). We investigated the appearance of  $\gamma\delta$  T cells in the peritoneal cavity after injection of LPS. Peritoneal cells were collected 3 or 5 days after i.p. injection of LPS. Peritoneal cells were stained directly with fluorescein isothiocyanate-conjugated an-



FIG. 7. Induction of HSP70 in peritoneal cells by administration of various preparations of LPS. Mice were injected i.p. with various preparations of LPS (100  $\mu$ g). Peritoneal cells were collected 4 days after injection. Lanes: 1, *S. enteritidis* LPS; 2, *K. pneumoniae* O3 LPS; 3, rough type LPS from *K. pneumoniae* O3; 4, free lipid A from *K. pneumoniae* O3. Molecular size markers in kilodaltons are on the left.

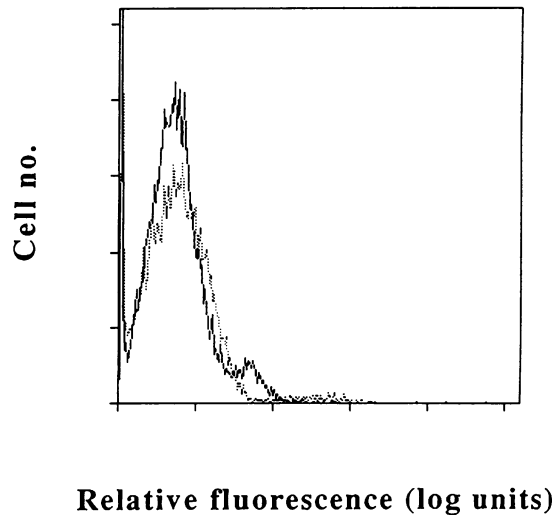


FIG. 8. Flow cytometric analysis of  $\gamma\delta$  T cells in the peritoneal cavities of mice injected i.p. with LPS. Mice were injected with LPS (100  $\mu$ g), and peritoneal cells were collected 5 days after injection. The histograms of untreated control mice (—) and LPS-treated mice (---) are shown. The fluorescence intensity is expressed on a log scale.

ti- $\gamma\delta$  T-cell receptor antibody (GL3), and the frequency of positively stained cells was analyzed by laser flow cytometry. The experimental results are shown in Fig. 8. No positive cells were detected in peritoneal cells from mice injected i.p. with LPS, although a small percentage of  $\gamma\delta$  T cells (approximately 10%) was found in peritoneal cells of untreated control mice.

#### DISCUSSION

In the present study, we have demonstrated that HSP70 was produced *in vivo* by peritoneal cells in mice injected i.p. with LPS. The evidence for this conclusion is based primarily on the induction of proteins electrophoretically and immunologically identical to HSP70. HSP70 might be produced by peritoneal macrophages in particular because it was detected only in the extract of adherent peritoneal cells from mice injected i.p. with LPS. No production of HSP70 in nonadherent peritoneal cells might be consistent with the failure of its production in thymocytes and spleen cells. Because purified LPS was injected, the HSP70 detected was definitely derived from the host cells and not from infectious agents. Therefore, it was concluded that LPS was a stressful agent which induces *in vivo* HSP responses in mice. HSP is induced in *in vivo* response to a range of different stresses, including heat, ischemia, reperfusion injury, wounding, and inflammation (15). To our knowledge, this is the first report that the i.p. administration of LPS can lead to the *in vivo* HSP response and the production of HSP70 in peritoneal macrophages of the host.

There are several reports that LPS causes an HSP response of cells in an *in vitro* culture system. Previously, it was reported that human monocytes cultured *in vitro* with LPS showed an increase of *in vitro* HSP70 transcripts and expression of HSP70 (2). On the other hand, it was found that the synthesis of the HSP70 family was reduced if the cells were cultured *in vitro* with LPS with heating at 43°C (18). It is still unclear how LPS affects the production of HSP even in an *in vitro* culture system of cells.

HSP70 is probably one of the best-studied stress proteins

found in all prokaryotes and eukaryotes, and it is, in particular, the most highly conserved of the HSP family. The highly inducible mammalian HSP70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon stress response (19, 24). Actually, HSP70 was undetectable at normal conditions in the present study but was highly inducible by injection of LPS. It has been proposed that HSP70 serves a role in the assembly and disassembly of protein complexes and translocation of certain proteins (6, 10, 24). It is possible that peritoneal macrophages produce HSP70 to preserve their cellular functions under the stressful condition of LPS administration.

On the basis of the observation that i.p. injection of LPS resulted in the *in vivo* production of HSP70, it is likely that production may require direct contact between the peritoneal macrophages and LPS at a relatively high concentration. It is also possible that the induction is a localized phenomenon at the site of injection. Furthermore, rough-type LPS induced more HSP70 than smooth-type LPS. At a given weight, rough-type LPS may contain more LPS molecules than smooth-type LPS. Physicochemical characteristics, such as solubility, of LPS preparations may also affect the induction of HSP70 through the interaction with macrophages.

The administration of TNF together with LPS markedly enhanced the expression of HSP70. This suggested that TNF cooperates synergistically with LPS because the *in vivo* administration of TNF alone did not cause the production of HSP. Since many effects of TNF are mediated by oxygen free radicals, oxygen free radicals might play some role in the enhancement of LPS-induced HSP induction by TNF (12, 15). From the present study, it is very likely that cytokines such as TNF may modulate the production of HSP70 in peritoneal macrophages by administration of LPS. The study of the action of cytokines on the *in vivo* production of HSP70 by LPS is now in progress.

T lymphocytes frequently recognize microbial HSP and could provide an early defense mechanism against pathogenic microbes. It was reported that certain  $\gamma\delta$  T cells seem to have a particular preference for this molecule (6–8). Skeen and Ziegler have reported that i.p. administration of LPS induced the increase in  $\gamma\delta$  T cells in the peritoneal cavity at day 3 after injection (17). In the present study, however, there was a decrease of  $\gamma\delta$  T cells in the peritoneal cavities of mice injected i.p. with LPS when HSP70 was expressed on adherent peritoneal cells. The frequency of  $\gamma\delta$  T cells might decrease somewhat because the injection of LPS results in the accumulation of a large number of polymorphonuclear leukocytes in the peritoneal cavity (23).

Recently, evidence that HSP are major antigens of many pathogens has been accumulating. The stress imposed by the host may lead to increased HSP synthesis by microorganisms. HSP becomes a prominent antigen that triggers a major portion of the immune repertoire. In particular, members of the HSP70 and HSP60 families are major targets for antibodies and T cells in many bacterial infections. On the other hand, the present study demonstrates that the stress imposed by LPS, a major component of the outer membrane of gram-negative bacilli, leads to the production of HSP by the host. Such host-derived HSP might also become the target of antibody or T cells directed toward bacterial HSP and could trigger an autoimmune reaction (21, 24, 25).

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