Recent studies have shown that commercially available recombinant human heat shock protein 60 (rhHSP60) could induce tumor necrosis factor α (TNF-α) release from macrophages and monocytes in a manner similar to that of lipopolysaccharide (LPS), e.g. via CD14 and Toll-like receptor 4 complex-mediated signal transduction pathway. In this study, we demonstrated that a highly purified rhHSP60 preparation with low endotoxin activity (designated rhHSP60-1) was unable to induce TNF-α release from murine macrophages at concentrations of up to 10 μg/ml. In contrast, a less purified rhHSP60 preparation (designated rhHSP60-2) was able to induce a marked TNF-α release at concentrations as low as 1 μg/ml. Failure of rhHSP60-1 to induce TNF-α release was not due to defective physical properties because rhHSP60-1 and rhHSP60-2 contained a similar amount of HSP60 as determined by SDS gels stained with Coomassie Blue and Western blots probed with an anti-rhHSP60 antibody. Both rhHSP60 preparations also had similar enzymatic activities as judged by their ability to hydrolyze ATP. Polymyxin B added in the incubation media abolished the endotoxin activity but inhibited only about 50% of the TNF-α-inducing activity of rhHSP60-2. However, both the endotoxin activity and the TNF-α-inducing activity of rhHSP60-2 were essentially eliminated after passing through a polymyxin B-agarose column that removes LPS and LPS-associated molecules from the rhHSP60 preparation. The TNF-α-inducing activities of both rhHSP60-2 and LPS with equivalent endotoxin activity present in rhHSP60-2 were equally sensitive to heat inactivation. These results suggest that rhHSP60 does not induce TNF-α release from macrophages. Approximately 50% of the observed TNF-α-inducing activity in the rhHSP60-2 preparation is due to LPS contamination, whereas the rest of the activity was due to the contamination of LPS-associated molecule(s).

The 60-kDa heat shock proteins (HSPs) (HSP60 or chaperonins) are highly conserved intracellular proteins expressed in all organisms, both constitutively and under stress conditions. The major function of members of the HSP60 family is to serve as molecular chaperones facilitating protein folding (1–3).

Members of the HSP60 family are classified into two groups (3). The group I chaperonins are best represented by the Escherichia coli GroEL-GroES complex. A functional unit of the E. coli chaperonin consists of two heptameric rings of identical GroEL (HSP60) subunits stacked back to back, and one heptameric ring of GroES (HSP10, the cofactor of GroEL) on one end of the stack. Protein folding occurs in the central cavity of the GroEL-GroES complex in an ATP-dependent manner. In eukaryotes, group I HSP60 has only been found in organelles of endosymbiotic origin, e.g. mitochondria and chloroplasts. Unlike GroEL, the mitochondrial HSP60 (mt-HSP60) exists in a dynamic equilibrium among monomers, heptamers, and tetradecamers (4). It dissociates into monomers at low concentrations and assembles into tetradecamers in the presence of ATP and mt-HSP10, the cofactor of mt-HSP60 (5). Together with mt-HSP70, the mt-HSP60-mt-HSP10 chaperonin complex plays an essential role in the folding of proteins after they are transported into mitochondria (6–8).

The group II chaperonins (cytosolic HSP60 or T-complex polypeptide-1) form heteroligomeric ring structures resembling that of the GroEL-GroES complex (1–3). The heteroligomeric ring of T-complex polypeptide-1, T-complex polypeptide-1 ring complex (TrcC), generally consists of eight or nine structurally related subunits of 52–65 kDa. It functions in cytosol to fold cytoskeletal proteins such as actin and tubulin (9, 10).

Considerable evidence suggests that HSP60 may also contribute to the pathogenesis of atherosclerosis. First, the expression of human and chlamydial HSP60 is increased in atherosclerotic plaques (11, 12). In apolipoprotein E-deficient mice, the expression of HSP60 at lesion-prone sites is temporally correlated with the development of atherosclerosis (13). Second, the level of circulating HSP60 is elevated in patients with hypertension and atherosclerosis (14, 15). Third, using recombinant chlamydial HSP60 and recombinant human HSP60 (rhHSP60), it has been shown that HSP60 induces the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 by monocytes and macrophages (12, 16–23), the expression of E-selectin by endothelial cells (21), and the proliferation of smooth muscle cells (23). These findings suggest that chlamydial infection and HSP60-induced inflammatory response may play important roles in the development of atherosclerosis (24–26).

Several studies (17–19, 22, 23) have shown that induction of pro-inflammatory cytokines by rhHSP60 is mediated through the CD14 and Toll-like receptor (TLR) 2- or TLR4-mediated signal transduction pathways. The CD14 and TLR complexes are pattern recognition receptors involved in the innate immunity for pathogen recognition and host defense (27, 28). CD14, the endotoxin (lipopolysaccharide (LPS)) receptor, is a glyco-

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From the †Institute for Clinical Research, ‡Veterans Affairs Medical Center, Washington, D. C. 20422 and §Georgetown University School of Medicine, Washington, D. C. 20057

1 The abbreviations used are: HSP, heat shock protein; rhHSP60, recombinant human HSP60; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; mt-HSP, mitochondrial HSP; TLR, Toll-like receptor; EU, endotoxin unit(s); LAL, Limulus Amebocyte Lysate.

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3 To whom correspondence should be addressed: VA Medical Center (10R), 50 Irving St., N.W., Washington, D. C. 20422. Tel.: 202-745-8000 (ext. 5426); Fax: 202-745-8538; E-mail: baochong.gao@med.va.gov.

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phosphatidylinositol-anchored membrane protein lacking transmembrane and intracellular domains (29, 30). TLRs are type I transmembrane proteins with an extracellular domain containing a leucine-rich repeat and a cytoplasmic domain analogous to that of the interleukin-1 receptor family (27, 31). Together with CD14, TLR4 initiates signaling cascades in response to LPS, whereas TLR2 initiates the signal cascades in response to bacterial lipoproteins and Gram-positive bacteria (27, 31–33). Thus, it has been suggested that HSP60, through its cytokine function, serves as a danger signal to the innate immune system (15) and that HSP60 could be the endogenous ligand for TLR2 and TLR4 (18, 22).

The reported cytokine function of HSP60 is unique, as compared with its molecular chaperone functions, in that it requires no ATP hydrolysis, no cofactor, and no protein complex assembly. In addition, it is similar to the function of bacterial products, LPS and lipoproteins. Because recombinant chlamydial HSP60 and rhHSP60 are produced by E. coli expressing chlamydia and human HSP60 CDNA, respectively, the final HSP60 preparations may be contaminated with bacterial products such as LPS and lipoproteins. Contamination with LPS and/or lipoproteins could be responsible for the reported cytokine function of rhHSP60s. In the current study, we demonstrate that rhHSP60 does not induce TNF-α release from murine macrophages and that the ability of a commercially available rhHSP60 preparation to induce TNF-α production by macrophages is due to contaminants in the rhHSP60 preparation.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human HSP60 proteins were purchased from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). The rhHSP60 was cloned from a human promyelocytic leukemia cDNA library and expressed in E. coli. Two preparations were available: catalog numbers ESP-540 (old catalog number ESP-740) and NSP-540 (old catalog no. SPP-740). The ESP-540 preparation was the low endotoxin preparation containing <50 endotoxin units (EU)/mg rhHSP60 as determined using Limulus Amebocyte Lysate (LAL) assay. This preparation did not contain GroEL and tested positive for ATCase activity. It was recommended for use in assays requiring low endotoxin. The NSP-540 preparation was not tested for endotoxin levels or ATCase activity. For the purpose of this report, the low endotoxin preparation, ESP-540, was designated rhHSP60-1, whereas the NSP-540 preparation was designated rhHSP60-2.

Phrotein-free LPS (from JM39 E. coli K-12, rough strain) was kindly provided by Dr. E. Sonervelle (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). Before use, LPS was dissolved in sterile pyrogen-free water, sonicated on ice for 30 s with a sonic dismembrator (Fisher Scientific, Houston, TX), and diluted with phosphate-buffered saline (Invitrogen). Polyoxynyl B sulfate (catalog number P4932; cell culture tested) and ATP were purchased from Sigma Chemical Co. Polyoxynyl B-agarose (Detoxi Gel; catalog number 20339) was obtained from Pierce. ECL Western blotting solution detection kits were purchased from Amersham Biosciences.

Cell Culture—RAW264.7 murine macrophages (from American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Subcultures of macrophages were prepared every 2 days by scraping cells into fresh medium.

Determination of TNF-α Release by Murine Macrophages—Murine macrophages were seeded in 24-well plates at 2.5 × 10⁶ cells/well the day before the experiment. After washing three times with the medium, the cells were treated with or without rhHSP60 (1–10 µg/ml) and/or LPS (0.05–0.4 ng/ml) in 250 µl of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum for 4 h at 37 °C. At the end of treatment, media were collected and clarified by centrifugation at 10,000 rpm for 5 min in a microcentrifuge (Hermle-Labortechnik, Weihingen, Germany). TNF-α content of the media was then determined by a quantitative sandwich enzyme-linked immunosorbent assay using an HRP-conjugated mouse TNF-α immunoassay kit (catalog number MTA00; R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendations. All experiments were carried out with duplicate samples.

In some experiments, rhHSP60 and LPS were heated for 60 min in a boiling water bath before being added to the cells. Likewise, in some experiments, macrophages were pre-incubated with or without polymyxin B sulfate (0.1–10 µg/ml) for 30 min at 37 °C before the addition of rhHSP60 or LPS to inactivate LPS.

Measurements of Endotoxin Activity—The endotoxin activities of rhHSP60 and LPS preparations were determined as described previously (34) using the LAL assay kit (catalog number 50-648U; BioWittaker, Walkersville, MD) according to the manufacturer’s recommendations.

Quantification of rhHSP60 Protein by Gel Electrophoresis and Western Blot—The rhHSP60 preparations and fractions from polymyxin B-agarose column (see below) were analyzed either by SDS-PAGE using 7.5% polyacrylamide gels followed by staining with Coomassie Blue or by Western blotting using an antibody to recombinant human HSP60 (clone 3B10; Cellomics Biotechnology Corp.) and visualized by ECL detection system as described previously (34, 35). Densitometric quantification of rhHSP60 of the Coomassie Blue-stained SDS gels or immunoblots was performed using the FluorChem 8000 digital imaging system (Alpha Innotech Corp., San Leandro, CA).

Assay for ATCase Activity of the rhHSP60 Preparations—The ATCase activity of rhHSP60 was determined as described previously (36). The buffer of HSP60-1 and HSP60-2 was changed into the ATPase Assay Buffer (50 mM HEPES, pH 7.4, 50 mM KC1, and 10 mM magnesium acetate) using Protein Desalting Spin Columns (Pierce) according to the manufacturer’s recommendations. 100 µl of each rhHSP60 preparation at 0.85 mg/ml was loaded onto a 700-µl desalting column. The recovery efficiency of the columns was 80–85% as determined by SDS-PAGE and densitometric scanning. rhHSP60 at 4 µM (240 µg/ml) was incubated with [γ-32P]ATP at 100 µM (specific activity, 0.1 Ci/mmol; ICN, Irvine, CA) at 30 °C. An aliquot of 25 µl was removed at 10, 25, and 40 min. The phosphate released from ATP hydrolysis was extracted into organic phase in orthophosphomolybdate complexes and quantified by liquid scintillation counting.

Removal of LPS and LPS-associated Molecules from rhHSP60-2 Using Polymyxin B-Agarose—LPS and LPS-associated molecules in rhHSP60-2 were removed using polymyxin B-agarose (Detoxi Gel; Pierce) as described previously (34). Briefly, aliquots of 0.5 ml of polymyxin B-agarose were poured into Poly-Prep disposable columns (Bio-Rad Laboratories, Hercules, CA). Columns were washed with 5 volumes of 1% sodium deoxycholate followed by 20 volumes of phosphate-buffered saline. 250 µl of rhHSP60-2 at 300 µg/ml was loaded onto each 0.5-ml Detoxi Gel column and incubated at room temperature for 60 min. The column was then eluted with phosphate-buffered saline in 250-µl fractions. The Detoxi Gel column fractions were analyzed by SDS-PAGE, and their rhHSP60 protein contents were determined by densitometric scanning.

Statistical Analysis—Results were expressed as mean ± S.D. Levels of significance were determined using a two-tailed Student’s t test (37), and a confidence level of >95% (p < 0.05) was used to establish statistical significance.

RESULTS

Induction of TNF-α Release from Murine Macrophages by rhHSP60 and LPS—As shown in Fig. 1A, rhHSP60-1 at 1 µg/ml, as compared with control, did not cause an increase in TNF-α release by murine macrophages. In contrast, rhHSP60-2 at the same concentration induced a significant increase in TNF-α release to an extent similar to that induced by 0.05 ng/ml LPS. Dose-response studies (Fig. 1B) revealed that rhHSP60-1 at concentrations of up to 10 µg/ml failed to induce TNF-α release. However, rhHSP60-2 at concentrations ranging from 1 to 10 µg/ml caused a dose-dependent increase in TNF-α release from murine macrophages.

Functional Properties and Endotoxin Contents of rhHSP60-1 and rhHSP60-2—There were two possible interpretations for the above-mentioned observations. First, rhHSP60 had no effect on the TNF-α release by macrophages; the observed effect of rhHSP60-2 was due to some contaminant(s) present in the rhHSP60-2 preparation. Second, rhHSP60 did have TNF-α-inducing effect; failure of rhHSP60-1 to induce TNF-α release by macrophages was due to the presence of functionally inactive rhHSP60 in the rhHSP60-1 preparation. To distinguish these two possibilities, we determined the functional characteristics as well as endotoxin contents of the rhHSP60-1 and rhHSP60-2 preparations.

As shown in Fig. 2, A and B, SDS-PAGE and immunoblots showed that rhHSP60-1 and rhHSP60-2 preparations contained approximately equal amounts of rhHSP60 protein. However, SDS-PAGE and immunoblots revealed that rhHSP60-2 preparations contained significantly more endotoxin than rhHSP60-1 preparations. Western blotting with an antibody to recombinant human HSP60 (clone 3B10; Cellomics Biotechnology Corp.) revealed that rhHSP60-1 and rhHSP60-2 preparations were equally contaminated with endotoxin (Fig. 2B). Therefore, we concluded that the observed effect was due to some contaminant(s) present in the rhHSP60-2 preparation. However, the HSP60-1 preparation failed to induce TNF-α release.
analyses of rhHSP60-1 and rhHSP60-2 revealed that both preparations had similar rhHSP60 protein content, molecular mass, and ability to interact with an anti-rhHSP60 antibody. HSP60 forms complexes with the cofactor HSP10, and the complex functions as a molecular chaperone in an ATP-dependent manner (5, 38). HSP60 also hydrolyzes ATP in the absence of protein folding, which is known as the uncoupled ATPase activity (5, 38). To determine whether both rhHSP60 preparations are functionally active, we measured the uncoupled ATPase activity of these two rhHSP60 preparations. To carry out the ATPase assay, the buffer of HSP60 preparations was changed into ATPase Assay Buffer using desalting columns. The protein concentration of HSP60 preparations after buffer exchange was determined by SDS-PAGE and densitometric scanning (Fig. 2C). The results revealed that rhHSP60-1 and rhHSP60-2 hydrolyzed ATP at similar rates over a time course of 40 min (Fig. 2D). The estimated rate of ATP hydrolysis was (2.89 ± 0.13) × 10^{-2} min^{-1} for rhHSP60-1 and (2.44 ± 0.17) × 10^{-2} min^{-1} for rhHSP60-2.

In contrast, rhHSP60-2 contained a markedly higher content of endotoxin than rhHSP60-1 as determined using the LAL assay. As shown in Fig. 3A, the endotoxin activity of rhHSP60-1 was 8.6 ± 3.9 EU/mg, whereas the endotoxin activity of rhHSP60-2 was 85.6 ± 20.6 EU/mg. The endotoxin activity of E. coli LPS was (2.7 ± 0.8) × 10^6 EU/mg. Thus, the endotoxin content in the rhHSP60-2 preparation was about 10 times that in the rhHSP60-1 preparation. The calculated equivalent LPS concentration in rhHSP60-1 was 3.2 pg/μg, whereas it was 32 pg/μg in the rhHSP60-2.

As shown in Fig. 1A, 1 μg/ml rhHSP60-2 with an endotoxin content equivalent to that of 32 pg/ml LPS induced a TNF-α release comparable with that induced by 50 pg/ml LPS. We therefore determined whether rhHSP60-2 with similar endotoxin activity was more potent than LPS in the induction of TNF-α release from macrophages. The results (Fig. 3B) showed that, indeed, at similar endotoxin activities, rhHSP60-2 induced approximately twice as much TNF-α release as that induced by LPS.
Role of LPS in TNF-α-inducing Activity of rhHSP60-2

The results shown in Fig. 3 suggested that in addition to LPS, factor(s) other than LPS in the rhHSP60-2 preparation might contribute to the observed TNF-α-inducing activity. Alternatively, rhHSP60 might enhance the potency of TNF-α-inducing activity of LPS without affecting its endotoxin activity as determined by the LAL assay.

To determine whether rhHSP60 selectively enhanced the TNF-α-inducing activity of LPS, we studied the effect of rhHSP60 on the endotoxin activity and TNF-α-inducing activity of LPS. In these experiments, LPS was preincubated with rhHSP60-1 for 40 min before being added to macrophages. As shown in Fig. 4, rhHSP60-1 at a concentration of 5 μg/ml had no effect on either the endotoxin activity (Fig. 4A) or the TNF-α-inducing activity (Fig. 4B) of LPS at concentrations ranging from 0.1 to 0.4 ng/ml.

The contribution of contaminating LPS to the endotoxin activity and TNF-α-inducing activity of rhHSP60-2 was further defined using a LPS-specific inhibitor, polymyxin B (39, 40). Two approaches were taken. First, polymyxin B was added directly to the macrophage incubation media to inhibit LPS activities. Second, polymyxin B-agarose (Detoxi Gel) column was used to remove LPS and LPS-associated molecules from rhHSP60-2. In the first approach, polymyxin B was added to macrophages 30 min before the addition of rhHSP60-2 or LPS. As shown in Fig. 5A, polymyxin B inhibited the endotoxin activity of both rhHSP60-2 (5 μg/ml) and LPS (0.2 ng/ml) to a similar extent, and a complete inhibition was achieved at a polymyxin B concentration of 10 μg/ml. In contrast, whereas the TNF-α-inducing activity of LPS (0.2 ng/ml) was completely inhibited by polymyxin B at a concentration as low as 0.1 μg/ml, only about 50% of the TNF-α-inducing activity of rhHSP60-2 was inhibited by polymyxin B even at a concentration of 10 μg/ml. These data suggest that the contaminating LPS contributed to 100% of the rhHSP60-2 endotoxin activity but only to about 50% of the rhHSP60-2 TNF-α-inducing activity.

In the second approach, the concentration of rhHSP60 in the fractions collected from polymyxin B-agarose columns was determined using SDS-PAGE (Fig. 6A) and densitometric quantification. The same amounts of rhHSP60 present in the polymyxin B-agarose column fractions as in the rhHSP60-2 preparation before passing through the column were then used to determine the endotoxin activity and the TNF-α-inducing activity. As shown in Fig. 6B, the polymyxin B-agarose column removed >90% of the endotoxin activity from rhHSP60-2. Likewise, polymyxin B-agarose also removed >90% of the TNF-α-
inducing activity of rhHSP60-2 (Fig. 6C).

The results above suggested that about 50% of the rhHSP60-2 TNF-α-inducing activity was due to the contaminating LPS, whereas the other 50% of the activity was due to factor(s) other than LPS that was bound to or associated with LPS. Both LPS and LPS-associated contaminants could be removed from rhHSP60-2 preparation by polymyxin B-agarose column.

Effect of Heat Inactivation on TNF-α-inducing Activities of LPS and rhHSP60-2—Heat sensitivity has been used in many studies (12, 17, 20, 21) to rule out the possibility that the observed effects were not due to the contaminating LPS in the rhHSP60 preparations. We have recently demonstrated (34) that contrary to popular belief, LPS is highly heat-sensitive, especially at low concentrations. A question remains regarding whether the non-LPS contaminant(s) in the rhHSP60-2 preparation that was responsible for 50% of the observed TNF-α-inducing activity was also heat-sensitive. We therefore determined the heat sensitivity of the endotoxin activity and the TNF-α-inducing activity of LPS and rhHSP60-2. As shown in Fig. 7, heating for 60 min in a boiling water bath almost completely inactivated the endotoxin activity as well as the TNF-α-inducing activity of both rhHSP60-2 and LPS. These data suggested that the non-LPS contaminant(s) responsible for 50% of the rhHSP60-2 TNF-α-inducing activity was also heat-sensitive.

DISCUSSION

The results presented in the current study demonstrated that rhHSP60 was unable to induce TNF-α release from murine macrophages and that the TNF-α-inducing activity of the rhHSP60-2 preparation was due to contaminants. This conclusion was derived from the following evidence. First, the highly purified rhHSP60 preparation (rhHSP60-1) with a low LPS content was unable to induce TNF-α release from macrophages at concentrations of up to 10 μg/ml, whereas the less purified rhHSP60 preparation (rhHSP60-2) was able to induce TNF-α release at concentrations as low as 1 μg/ml (Fig. 1). Second,
failure of rhHSP60-1 to induce TNF-α release was not due to
defective physical properties because rhHSP60-1 and
rhHSP60-2 contained identical HSP60 content as determined
by SDS gels stained with Coomassie Blue and Western blots
probed with an anti-rhHSP60 antibody. Both rhHSP60 prepa-
rations also had similar enzymatic activity as judged by their
ATPase activities (Fig. 2). Third, removal of LPS and LPS-
associated factor(s) by polymyxin B-agarose column completely
eliminated the TNF-α-inducing activity of rhHSP60-2 (Fig. 6).

We believed that ~50% of the rhHSP60-2 TNF-α-inducing
activity was due to LPS contamination, whereas the other 50%
was due to non-LPS contaminant(s) that was closely associated
with LPS. This conclusion was based on the following observa-
tions. First, the TNF-α-inducing activity of rhHSP60-2 was
twice as much as that of LPS with equivalent endotoxin activ-
ity (Fig. 3). Because rhHSP60-1 did not enhance LPS TNF-α-
inducing activity (Fig. 4), factor(s) other than LPS was respon-
sible for half of the rhHSP60-2 TNF-α-inducing activity.

Second, polymyxin B could inhibit only 50% of the rhHSP60-2
TNF-α-inducing activity, whereas it inhibited essentially 100%
of the endotoxin activity of rhHSP60-2 (Fig. 5), suggesting that
the contaminating LPS was responsible for 50% of the TNF-α-
inducing activity but 100% of the endotoxin activity of
rhHSP60-2. On the other hand, polymyxin B-agarose column
removed nearly 100% of rhHSP60-2 TNF-α-inducing activity as
well as endotoxin activity, suggesting that LPS-associated fac-
tor(s) was responsible for the other 50% of rhHSP60-2 TNF-α-
inducing activity. We further showed that this contaminating
LPS-associated factor(s) was heat-sensitive (Fig. 7).

The exact nature of this LPS-associated factor(s) is not clear.
However, LPS-associated proteins such as lipoproteins are po-
tential candidates. Bacterial lipoproteins are potent activators
of monocytes and macrophages capable of inducing TNF-α pro-
duction (41, 42). It has been shown that LPS
lipoprotein complexes in the LPS preparation are responsible for the activation
of LPS-unresponsive C3H/HeJ spleen cells (43, 44) and that the
activity of these complexes is not affected by polymyxin B
added directly to the culture media (43, 45). Contamination of
rhHSP60 with LPS and bacterial lipoproteins might explain
the previous observation that rhHSP60 induces TNF-α release
from macrophages via TLR2- as well as TLR4-mediated signal
transduction pathways (22).

Using rhHSP60 obtained from the same source as the one we
used in the current study (e.g. StressGen Biotechnologies
Corp.), a number of laboratories have reported that rhHSP60
induces a marked production of TNF-α or interleukin-6 by
monocytes and macrophages (12, 16 – 22). However, none of
these reports indicate which rhHSP60 preparation from Stress-
Gen Biotechnologies Corp. was used in the studies. Some of
these studies made an effort to rule out LPS contamination as
being responsible for the observed cytokine-inducing activity of
rhHSP60 using two criteria. First, they showed that the cyto-
kine-inducing activity of rhHSP60 was heat-sensitive, whereas
that of LPS was heat-resistant. However, most studies (12, 17,
20, 21) used LPS at much higher concentrations (e.g. >100
g/ml) than those present in the rhHSP60 preparations. Be-
cause LPS is capable of inducing TNF-α production by macro-
phages at concentrations of <1 ng/ml, even if the heat treat-
ment inactivated >99% of the LPS used in their studies, the
residual LPS would still be able to induce TNF-α production,
thus giving the impression that LPS is heat-resistant. As we
have demonstrated previously (34) and again here in the cur-

Fig. 7. Effect of heat inactivation on endotoxin activities and TNF-α-inducing activities of rhHSP60-2 and LPS. Stock solutions of
rhHSP60-2 at 100 μg/ml and LPS at 4 ng/ml were heated in boiling water bath for 1 h. Endotoxin activities of non-heated rhHSP60-2 and heated
rhHSP60-2 (A) or non-heated LPS and heated LPS (B) were determined. Macrophages were treated with rhHSP60-2, heated rhHSP60-2, LPS, or
heated LPS at the indicated concentrations for 4 h. TNF-α concentrations in media were then determined (C and D). Values represent means ±
S.D. of three experiments. *, p < 0.05 (versus non-heated controls).
rent study, LPS at concentrations similar to those present in contaminating rhHSP70 and rhHSP60 is as heat-sensitive as the rhHSPs. The second criterion used in these studies was the sensitivity of the cytokine-inducing activity of rhHSP60 to polymyxin B. These studies found either no or incomplete inhibition of rhHSP60 cytokine-inducing activity by polymyxin B. However, as demonstrated in the current study, the StressGen Biotechnologies Corp. rhHSP60-2 preparation contains, in addition to LPS, other non-LPS contaminant(s) that was capable of inducing TNF-α production but was insensitive to polymyxin B.

Ample examples exist in the literature demonstrating how contaminants can lead to misleading conclusions. For example, in 1998, using a commercially available LPS preparation, it was first reported that TLR2 mediated LPS-induced cell activation of nuclear factor κB and could be the long sought after LPS signal transducer (47–49). In 2000, it was then demonstrated that TLR2 could not mediate cellular response by commercial preparations of LPS when these LPS preparations were re-purified (50, 51). In 2002, two lipoproteins (Lip12 and Lip19) extracted from E. coli LCD25 LPS were identified to be the major components responsible for the TLR2-mediated cell activation in the commercial LPS preparations (52). Thus, failure to recognize the presence of lipoproteins in the commercially available LPS preparation led to the erroneous attribution of lipoprotein signal transducer, TLR2, as the LPS signal transducer.

In view of the results presented in the current study as well as our previous study demonstrating that LPS contamination in commercially available rhHSP70 preparation was responsible for the induction of TNF-α release by murine macrophages, we conclude that HSP60 and HSP70 do not induce TNF-α production by macrophages. Recently, Bausinger et al. (53) also reported that endotoxin-free HSP70 fails to induce antigen-presenting cell activation. Therefore, the vast literature reporting the cytokine function of rhHSP60 and rhHSP70 in recent years needs careful scrutiny.

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