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# Mycobacterial 65-Kilodalton Heat Shock Protein Induces Tumor Necrosis Factor Alpha and Interleukin 6, Reactive Nitrogen Intermediates, and Toxoplasmastatic Activity in Murine Peritoneal Macrophages

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**The 65-kDa heat shock protein (Hsp65) is supposed to play a role in host defense against infections with various microbial pathogens and in autoimmune inflammatory disorders. These effects are thought to result mainly from an Hsp65-specific T-lymphocyte-mediated immune response that recognizes conserved epitopes. The aim of the present study was to assess whether mycobacterial Hsp65 has a direct effect on resident murine peritoneal macrophages, independent of Hsp65-sensitized T lymphocytes. Exposure of peritoneal macrophages from naive C57BL/6 mice to the mycobacterial Hsp65 in vitro induced an enhanced release of tumor necrosis factor alpha (TNF-α) and interleukin 6. These cells also produced large amounts of reactive nitrogen intermediates (RNI) and inhibited the intracellular proliferation of *Toxoplasma gondii*. Small amounts of gamma interferon acted synergistically with Hsp65. Thus, exposure of murine macrophages to Hsp65 results in activation of these cells. The acquisition of these characteristics by peritoneal macrophages occurred in the absence of sensitized T lymphocytes. Addition of anti-TNF-α antiserum resulted in an attenuation of the Hsp65-induced release of RNI and toxoplasmastatic activity, indicating that endogenous TNF-α is involved in the Hsp65-induced macrophage activation. The conclusion of this study is that in vitro exposure of peritoneal macrophages to the mycobacterial Hsp65 induces the release of proinflammatory cytokines and RNI and results in inhibition of the intracellular proliferation of *T. gondii*. These effects on murine macrophages occur independently of Hsp65-specific T lymphocytes. The proinflammatory effect of Hsp65 demonstrated in this study suggests that this heat shock protein may play a role in the initiation of inflammation that adds to a non-species-specific resistance in the early stages of infections.**

Heat shock proteins are constitutively present in eucaryotic and procaryotic cells. The production of heat shock proteins is greatly enhanced by stress stimuli such as a rise in temperature, exposure to toxic oxygen radicals, and nutritional deficiencies. The main function of heat shock proteins is preservation of essential cellular proteins and functions. The mycobacterial 65-kDa heat shock protein (Hsp65) belongs to the major 60-kDa heat shock protein family that displays a high degree of genotypic and phenotypic homology among various microorganisms and the human counterpart (12, 13, 17, 28, 34). Hsp65 is an immunodominant antigen that elicits a strong humoral and cellular immune response. Hsp65 may participate in the host defense against invading microorganisms and may also play a role in immune inflammatory disorders such as arthritis, diabetes mellitus, and Crohn's disease. These effects are thought to result mainly from an Hsp65-specific T-lymphocyte-mediated immunity that recognizes conserved epitopes (9, 15, 16, 24, 25, 31). Recently, it has been shown that in vitro exposure of human monocytes and monocyte-derived macrophages to mycobacterial Hsp65 induces the release of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β). The enhanced release of proinflammatory cytokines by these cells occurred in the absence of Hsp65-sensitized T lymphocytes (11, 26). TNF-α, together with gamma interferon (IFN-γ), can act as an autocrine signal to augment the microbicidal effector functions of macrophages (18, 19, 21).

Hsp65 homologs are abundantly expressed in inflammatory lesions and produced by microorganisms during invasive infection and phagocytosis (6, 24). Our hypothesis is that Hsp65 is involved in the pathogenesis of various inflammatory diseases and adds to a first-line host defense against invasive pathogens by its capacity to induce the endogenous production of proinflammatory cytokines, which in turn activate antimicrobial functions of mononuclear phagocytes. The aim of the present study was to assess whether exposure of murine macrophages to Hsp65 has a direct effect on the functions of these cells, independent of Hsp65-sensitized T lymphocytes.

## MATERIALS AND METHODS

**Animals.** Specific-pathogen-free female C57BL/6 mice weighing between 20 and 25 g were purchased from IFFA-Credo, Saint-Germaine sur l'Abresle, France. The animals were maintained in laminar horizontal flow cabinets, and sterilized food and tap water were given ad libitum.

**Hsp65.** Recombinant mycobacterial Hsp65, kindly provided by J. D. A. van Embden, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, was produced and purified as described earlier (27, 29). The protein was suspended in pyrogen-free saline (pH 7.4) to a concentration of 1 mg/ml. Endotoxin (lipopolysaccharide; LPS) (>1 mg of LPS per mg of protein) was removed from the suspension by means of affinity chromatography with Affi-Prep polymyxin B-coated macroporous beads (Bio-Rad, Richmond, Calif.). The residual LPS content was <0.8 ng per mg of protein as determined by *Limulus* lysate assay.

**Cytokines and antibodies.** Rat recombinant IFN-γ (rIFN-γ) with a specific activity of $4 \times 10^6$ U/mg of protein, produced in Chinese hamster ovary cells, was kindly provided by P. H. van der Meide (Biological Primate Research Center, Rijswijk, The Netherlands) (30). Lyophilized rIFN-γ was diluted in pyrogen-free

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saline and stored at −70°C, and aliquots were thawed only once, immediately before use. The LPS content of these solutions was <0.35 ng per mg of protein.

Aliquots of a rabbit anti-mouse TNF-α antiserum (generously provided by Innogenetics, Ghent, Belgium) containing approximately $10^6$ neutralizing units per ml and of normal rabbit serum as controls were stored at −70°C and thawed immediately before use. Neutralizing monoclonal antibody specific for rat and mouse IFN-γ (DB1) (30) containing $5 \times 10^3$ neutralizing units per mg (gift from P. H. van der Meide) was stored in aliquots at −70°C and thawed immediately before use.

**Isolation and stimulation of peritoneal macrophages.** Resident peritoneal macrophages were harvested by lavage of the peritoneal cavities of mice with 2 ml of ice-cold phosphate-buffered saline (PBS) containing 50 U of heparin per ml as described previously (32). The cells were washed with ice-cold PBS containing 0.5 U of heparin per ml and counted in a Bürker hemocytometer. The macrophages were suspended to a concentration of $10^6$/ml in sterile LPS-free RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum (Gibco BRL, Paisley, United Kingdom), 1,000 U of sodium penicillin G (Brocades Pharma B.V., Leiderdorp, The Netherlands) per ml, 50 μg of streptomycin (Biochemie GmbH, Vienna, Austria) per ml, and 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland), hereafter called medium. The LPS content of the medium was <0.08 ng/ml. One milliliter of the cell suspension was plated on 35-mm-diameter tissue culture dishes (Falcon, Plymouth, England) containing three 12-mm-diameter round glass coverslips and incubated for 1 h at 37°C and 7.5% $CO_2$. Nonadherent cells were removed by washing, and the adherent macrophages were incubated with 1 ml of medium containing 50, 10, 1, or 0.1 μg of Hsp65, 10 μg of ovalbumin, or 100 U of rIFN-γ plus 10 ng of *Escherichia coli* LPS (O11:B4; Sigma, St. Louis, Mo.). After incubation for 3 and 24 h, samples were taken from the cell culture supernatants and processed immediately. After incubation for 24 h, the intracellular proliferation of *Toxoplasma gondii* in the macrophages was assessed.

**Measurement of cytokine production.** The production of TNF-α was determined by means of an enzyme-linked immunosorbent assay (ELISA). A 96-well Nunc-Immuno plate (Nunc, Roskilde, Denmark) was coated overnight at 4°C with 2 μg of rat anti-mouse TNF-α monoclonal antibody (Pharmingen, San Diego, Calif.) per ml in a volume of 100 μl of coating buffer (25 mM $Na_2CO_3$, 25 mM $NaHCO_3$, 0.02% sodium azide, pH 9.6). The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) and next incubated for 2 h at room temperature with 200 μl of PBS containing 10% heat-inactivated newborn calf serum (Gibco) (PBS-$NBCS_i$). After the wells were washed three times, 100 μl of cell-free supernatant sampled after 3 h of incubation of the macrophages or 100 μl of standard rTNF-α dilutions was added in triplicate and incubated overnight at 4°C. The wells were washed with PBS-Tween and subsequently incubated with 1 μg of biotinylated rat anti-mouse TNF-α monoclonal antibody per ml in a volume of 100 μl of PBS-$NBCS_i$ during 45 min at room temperature. After being washed, the wells were incubated with 100 μl of PBS-$NBCS_i$ containing 0.25 μg of streptavidin-peroxidase (Sigma) per ml during 1 h at room temperature. The wells were thoroughly washed, and 100 μl of sodium-acetate buffer (pH 6.0) containing 0.02% (vol/vol) $H_2O_2$ and 0.1 mg of 3,3′,5,5′-tetramethylbenzidine (Sigma) per ml was added. After 15 min of incubation at room temperature, the reaction was stopped and $A_{450}$ were read on a Titertek Multiscan Plus (EFLAB, Helsinki, Finland).

The level of production of IFN-γ was determined by ELISA (generously provided by P. H. van der Meide). In short, a 96-well plate (Titertek Immunoassay plate; Flow Laboratories) was coated overnight at 4°C with 15 μg of anti-IFN-γ (DB1) per ml in a volume of 100 μl of PBS (pH 8.2). After being washed with Tris-buffered saline containing 0.05% Tween 20, the wells were incubated with 200 μl of PBS containing 2% bovine serum albumin. Next, 100 μl of cell-free supernatant sampled after 24 h of incubation of the macrophages or standard rat IFN-γ dilutions (Biological Primate Research Center) was added after washing and incubated for 2 h at 37°C. After the wells were washed, 100 μl of PBS containing 60 ng of biotinylated rabbit anti-rat IFN-γ antibodies was added and the wells were incubated for 1 h at 37°C; next, the wells were washed and 100 μl of saline containing 250 mU of anti-biotin alkaline phosphatase (Boehringer GmbH, Mannheim, Germany) was added. After incubation for 1 h at 37°C, the wells were washed, 100 μl of substrate buffer (1 M glycine, 10 mM $ZnCl_2$, 10 mM $MgCl_2$) containing 50 μg of *p*-nitrophenylphosphate (Sigma) was added, and the wells were incubated for 30 min at 37°C. After the addition of 30 μl of 3 N NaOH, the $A_{405}$ was read on a Titertek Multiscan Plus (EFLAB).

The production of IL-6 by peritoneal macrophages was assessed by the B-9 bioassay (33).

**Measurement of nitrite production.** The amount of nitrite ($NO_2^-$) released by peritoneal macrophages after 24 h of incubation was measured in cell-free supernatants by the colorimetric Griess reaction as previously described (1, 8). In short, 50 μl of the supernatant was mixed with 50 μl of Griess reagent consisting of 1% sulfanylamide, 0.1% naphthylethylene-diamide-dihydrochloride, and 2.5% $H_3PO_4$ and incubated for 10 min at room temperature. $A_{550}$ of the reaction product reflecting the concentration of $NO_2^-$ were read on a Titertek Multiscan Plus. All measurements were performed in triplicate, and the results are expressed in nanomoles of $NO_2^-$ per $10^6$ macrophages.

**Intracellular proliferation of *T. gondii*.** The virulent RH strain of *T. gondii* was maintained by biweekly intraperitoneal passage in mice. After collection of the tachyzoites by peritoneal lavage, peritoneal macrophages were infected with *T. gondii* as described previously (18, 25). In brief, macrophages were suspended in medium at a concentration of $10^6$/ml. Two milliliters was plated on 35-mm-diameter plastic culture dishes (Falcon) containing three 12-mm-diameter round glass coverslips and incubated for 2 h at 37°C and 7.5% $CO_2$. Nonadherent cells were removed by washing. Next, the adherent macrophages were incubated with 1 ml of medium containing $10^6$ toxoplasmas per ml for 30 min at 37°C and 7.5% $CO_2$ and then washed three times with warm PBS to remove noningested toxoplasmas. The experimental conditions were chosen so that 25 to 30% of the macrophages would be infected. One coverslip was fixed in methanol and stained with Giemsa stain for determination of the number of ingested protozoa and the percentage of infected macrophages at the start of the assay. Fresh medium was added to the cells on the other coverslips, and they were incubated for another 18 h at 37°C and 7.5% $CO_2$. After fixation of the cells in methanol and staining with Giemsa stain, the number of toxoplasmas per 100 macrophages was determined microscopically. The results of the assay are expressed as the fold increase, i.e., the ratio of the mean number of toxoplasmas per 100 macrophages after 18 h of incubation to the mean number of toxoplasmas per 100 macrophages at 0 h.

**Statistical analysis.** The results are expressed as means ± standard deviations of at least four independent experiments. The significance of differences in values was assessed by Student's paired *t* test. The level of significance was set at 0.05.

## RESULTS

**Production of proinflammatory cytokines by peritoneal macrophages after in vitro stimulation with Hsp65.** The amount of TNF-α produced by $10^6$ peritoneal macrophages of C57BL/6 mice after 3 h of incubation with 10 and 50 μg of Hsp65 per ml in vitro was significantly higher than that produced by peritoneal macrophages incubated with 10 μg of ovalbumin per ml or incubated in medium only (referred to as the controls) ($P < 0.005$ and $P < 0.001$, respectively) (Fig. 1A). Lower concentrations of Hsp65, i.e., 1 and 0.1 μg per ml, did not ($P > 0.05$) induce an enhanced production of TNF relative to that produced by control macrophages. Ovalbumin, which served as a control protein, did not ($P > 0.2$) induce an enhanced production of TNF-α by macrophages compared with the result for macrophages incubated in medium only.

Since IFN-γ with or without LPS stimulates macrophages to release proinflammatory cytokines (18), the release of TNF-α by peritoneal macrophages stimulated in vitro with 100 U of IFN-γ per ml together with 10 ng of LPS per ml was assessed and compared with Hsp65 stimulation. The amount of TNF-α produced by IFN-γ-and-LPS-stimulated peritoneal macrophages was not significantly different ($P > 0.05$) from that produced by peritoneal macrophages stimulated with 10 or 50 μg of Hsp65 per ml but was significantly ($P < 0.001$) enhanced relative to that produced by the controls (Fig. 1A).

Peritoneal macrophages that were incubated for 24 h with 50 μg of Hsp65 per ml produced significantly ($P < 0.01$) more IL-6 than peritoneal macrophages incubated with ovalbumin or incubated in medium only. In vitro incubation of peritoneal macrophages with 10 μg of Hsp65 per ml or with IFN-γ together with LPS also resulted in an enhanced production of IL-6 compared with that produced by the controls (Fig. 1B). The amount of IL-6 released by macrophages during stimulation with IFN-γ together with LPS was similar to that released during stimulation with 10 or 50 μg of Hsp65 per ml. Ovalbumin did not induce an enhanced production of IL-6 compared with that produced by macrophages incubated in medium only.

Incubation of the cells with 10 μg of Hsp65 per ml for 24 h did not induce detectable amounts of IFN-γ.

**Production of $NO_2^-$ by peritoneal macrophages after in vitro stimulation with Hsp65.** During in vitro incubation with 10 and 50 μg of Hsp65 per ml for 24 h, peritoneal macrophages produced significantly ($P < 0.005$) more $NO_2^-$ than they produced during incubation with ovalbumin or in medium only (Table 1). The amount of $NO_2^-$ produced by peritoneal macrophages incubated with 1 or 0.1 μg of Hsp65 per ml did not differ from that produced by the controls ($P > 0.05$). Ovalbumin did not induce an enhanced production of $NO_2^-$ by mac-

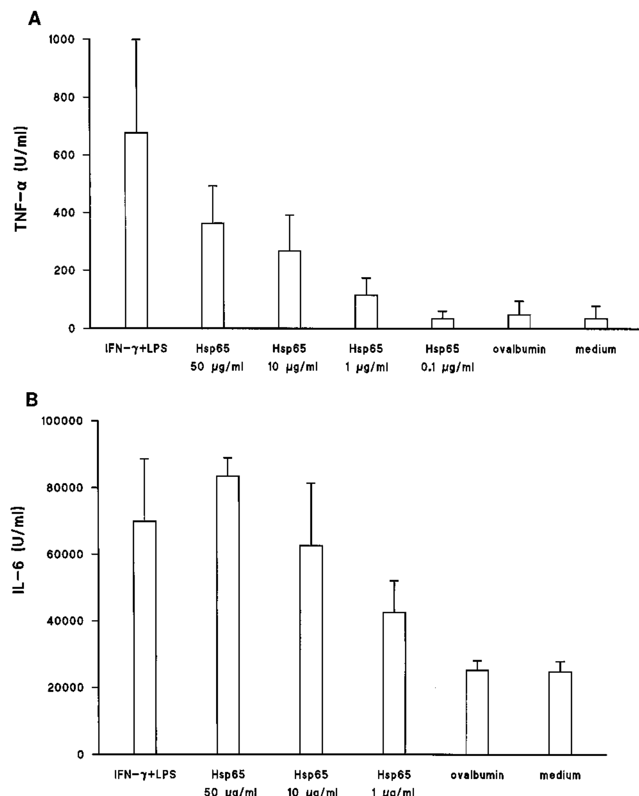


FIG. 1. Production of TNF-α (A) and IL-6 (B) by peritoneal macrophages during incubation with Hsp65, IFN-γ together with LPS, ovalbumin, or in medium only. The amounts of TNF-α and IL-6 in the supernatant were determined after 3 and 24 h of incubation, respectively. Data are means ± standard deviations of six independent experiments for TNF-α and of four independent experiments for IL-6.

rophages, compared with that produced by macrophages incubated in medium only.

In vitro incubation of peritoneal macrophages with IFN-γ together with LPS resulted in a larger production of $NO_2^-$ compared with that of macrophages stimulated with Hsp65 ($P < 0.01$) and that of the controls (Table 1). Small amounts of

TABLE 1. Release of $NO_2^-$ and intracellular proliferation of *T. gondii* in peritoneal macrophages incubated with Hsp65, IFN-γ and LPS, ovalbumin, or in medium alone

| Macrophages incubated in[a]: | Release of $NO_2^-$ [b] | Proliferation of *T. gondii*[c] |
|---|---|---|
| Medium | 8.9 ± 8.8 | 5.2 ± 1.0 |
| Ovalbumin | 6.6 ± 8.2 | 5.2 ± 1.5 |
| Hsp65 (μg/ml) | | |
| 50 | 30.7 ± 2.8*[d] | 2.0 ± 1.1* |
| 10 | 25.7 ± 9.0* | 3.2 ± 1.1* |
| 1 | 8.8 ± 9.7 | 5.6 ± 1.7 |
| 0.1 | 11.8 ± 11.8 | 5.1 ± 0.1 |
| IFN-γ and LPS | 62.4 ± 7.0* | 1.2 ± 0.5* |

[a] Peritoneal macrophages were incubated in vitro during 24 h.
[b] Data are expressed in nanomoles per $10^6$ macrophages. Results are means ± standard deviations of six independent experiments.
[c] Data are expressed as fold increase in the number of *T. gondii* per 100 macrophages at 18 h. Results are means ± standard deviations of six independent experiments.
[d] *, significant ($P < 0.05$) difference from results for control macrophages incubated with ovalbumin or in medium only.

TABLE 2. Effect of IFN-γ on the Hsp65-induced release of $NO_2^-$ by peritoneal macrophages[a]

| Macrophages incubated in: | Release of $NO_2^-$ [b] |
|---|---|
| Medium | 1.8 ± 1.7 |
| IFN-γ (100 U/ml) | 45.0 ± 15.0*[c] |
| IFN-γ (0.1 U/ml) | 4.1 ± 4.5 |
| Hsp65 (10 μg/ml) | 16.8 ± 5.0 |
| Hsp65 (10 μg/ml) plus IFN-γ (0.1 U/ml) | 34.0 ± 2.2* |
| Hsp65 (1 μg/ml) | 4.1 ± 2.8 |
| Hsp65 (1 μg/ml) plus IFN-γ (0.1 U/ml) | 16.1 ± 1.0* |

[a] Peritoneal macrophages were incubated in vitro during 24 h with Hsp65 in the presence or absence of a nonactivating concentration of IFN-γ.
[b] Data are expressed in nanomoles per $10^6$ macrophages. Results are means ± standard deviations of three independent experiments.
[c] *, significant ($P < 0.05$) increase in release of $NO_2^-$ relative to that of the controls incubated without IFN-γ.

IFN-γ acted synergistically with Hsp65 in the induction of $NO_2^-$ by the peritoneal macrophages (Table 2).

To exclude the possibility that some of the effects of Hsp65 on macrophages are mediated by contaminating cells, e.g., NK cells, that might produce IFN-γ upon incubation with the heat shock protein, neutralizing antibodies against IFN-γ were added. During incubation of macrophages with 10 μg of Hsp65 in the presence of 100 μg of anti-IFN-γ per ml, the amount of $NO_2^-$ (16.6 ± 3.0 nmol/$10^6$ cells; $n = 4$) released was the same as that released during incubation of macrophages with 10 μg of Hsp65 per ml alone (16.8 ± 5.0 nmol/$10^6$ cells; $n = 4$). Furthermore, no IFN-γ could be detected in the supernatants after 24 h of incubation with 10 μg of Hsp65 per ml.

**Intracellular proliferation of *T. gondii* in Hsp65-stimulated macrophages.** In vitro incubation of peritoneal macrophages during 24 h with 10 or 50 μg of Hsp65 per ml resulted in a significant ($P < 0.05$) inhibition of the intracellular proliferation of *T. gondii* compared with that for macrophages incubated with ovalbumin or incubated in medium only (Table 1). Lower concentrations of Hsp65 did not inhibit the intracellular proliferation of *T. gondii* compared with the case for the controls ($P > 0.1$). The intracellular proliferation of *T. gondii* in peritoneal macrophages incubated with ovalbumin and that in peritoneal macrophages incubated in medium only were similar. Incubation of peritoneal macrophages with IFN-γ together with LPS also resulted in inhibition of the intracellular proliferation of *T. gondii*, and the level of inhibition in this case was similar to that for peritoneal macrophages incubated with 50 μg of Hsp65 per ml ($P > 0.1$).

Treatment of macrophages with Hsp65 did not affect the ability of *T. gondii* to invade the cells. The percentages of infected macrophages at the start of the assay after treatment with 50 μg of Hsp65 per ml (23% ± 4%) and 10 μg of Hsp65 per ml (30% ± 4%) were similar ($P > 0.5$) to that of ovalbumin-treated macrophages (28% ± 6%) and macrophages incubated in medium only (26% ± 4%). Also, the total number of tachyzoites at the start of the assay did not differ between the various treatment groups, i.e., 29 ± 8, 36 ± 7, 33 ± 9, and 33 ± 7 tachyzoites per 100 macrophages treated with 50 μg of Hsp65 per ml, 10 μg of Hsp65 per ml, and 10 μg of ovalbumin per ml and incubated in medium only (controls), respectively.

**Role of TNF-α in Hsp65-induced macrophage activation.** Since TNF-α has been demonstrated to play an essential role in macrophage activation, we assessed the role of endogenously produced TNF-α in the Hsp65-induced macrophage activation. Neutralization of TNF-α by the addition of $10^4$ neutralizing units per ml resulted in a significant ($P < 0.02$) decrease in the Hsp65-induced production of reactive nitrogen

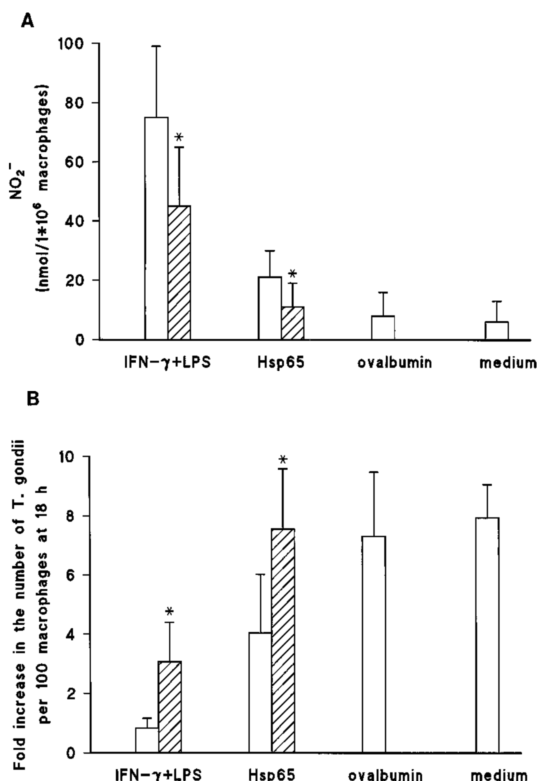


FIG. 2. Production of $NO_2^-$ by (A) and intracellular proliferation of *T. gondii* in (B) peritoneal macrophages after in vitro incubation with 50 μg of Hsp65 per ml, 100 U of IFN-γ per ml together with 10 ng of LPS, or 10 μg of ovalbumin per ml or in vitro incubation in medium only in the presence of neutralizing rabbit anti-TNF-α antiserum ($10^4$ neutralizing units per ml) (hatched bars) or an equivalent amount of normal rabbit serum (open bars). Results are means ± standard deviations of four independent experiments. *, significant ($P < 0.05$) difference compared with the result for stimulation without antiserum against TNF-α.

intermediates (RNI) and the inhibition of *T. gondii* proliferation relative to that seen after incubation with Hsp65 in the presence of normal rabbit serum (Fig. 2). The production of $NO_2^-$ and the toxoplasmastatic activity of macrophages stimulated with Hsp65 in the presence of anti-TNF-α antibodies were similar to those for controls ($P > 0.05$).

## DISCUSSION

The main conclusions from this study are that Hsp65 by itself induces activation of murine peritoneal macrophages, as judged by their ability to produce TNF-α, IL-6, and RNI and to inhibit the intracellular proliferation of *T. gondii*. This Hsp65-induced macrophage activation is, at least in part, mediated by endogenously produced TNF-α, since it is attenuated by neutralization of this cytokine. These results are obtained in the absence of sensitized T lymphocytes and represent a direct effect of Hsp65 on murine peritoneal macrophages. Incubation of peritoneal macrophages with ovalbumin, which served as a control protein, does not lead to macrophage activation, whereas incubation with IFN-γ together with LPS induces functional characteristics in peritoneal macrophages similar to those induced by incubation with Hsp65.

The effects of Hsp65 on peritoneal macrophages cannot be attributed to contamination with LPS. The amount of LPS needed for a similar release of TNF-α, increased toxoplasmastatic activity, and production of RNI by macrophages is much larger than that present in our preparations (5, 23). Even in the synergistic combination with IFN-γ, a much higher LPS concentration was required to obtain comparable results. Moreover, others have shown that Hsp65 induces an LPS-independent enhancement of TNF gene expression and secretion of the protein by human THP-1 monocytic cells and that a concentration of at least 10 μg of LPS per ml is required to obtain similar results (11).

Our finding that antiserum to TNF-α significantly reduced the Hsp65-induced production of nitrite and toxoplasmastatic activity of murine macrophages is in agreement with earlier studies demonstrating that endogenous TNF-α is required for the production of RNI and subsequent induction of toxoplasmastatic activity in murine peritoneal macrophages (7, 18, 19). Thus, it is suggested that Hsp65 directly induces the production of TNF-α, which in turn leads to RNI production and enhanced antimicrobial activity of the macrophages.

Hsp65 induces the production of IL-6, and it has been demonstrated recently that this cytokine enhances the intracellular proliferation of *T. gondii* (3). However, this effect of IL-6 on *T. gondii* proliferation is abrogated when TNF-α is added simultaneously (3). Hsp65 induces the production of both TNF-α and IL-6, which explains why *T. gondii* proliferation is inhibited despite the presence of large amounts of IL-6.

The observation that exposure to Hsp65 induces the secretion of the proinflammatory cytokines TNF-α and IL-6, increases the production of RNI, and inhibits the intracellular proliferation of *T. gondii* in peritoneal macrophages independently of sensitized T lymphocytes is surprising, since it is generally accepted that the effect of Hsp65 on host defense and autoimmune inflammatory disorders is due to an Hsp65-specific T-lymphocyte-mediated immune response. The latter view is not contradicted by our findings, but our results indicate that in addition to a T-lymphocyte-mediated immune reactivity to Hsp65, a direct effect of the exposure of mononuclear phagocytes to Hsp65 must be considered in the pathogenesis of inflammation.

Although we did not study the effects of Hsp65 derived from sources other than mycobacteria, this protein is highly conserved and has a high degree of homology among numerous microorganisms and even mammals (13, 28, 34). Expression of Hsp65 by various microorganisms is greatly enhanced in the cases of phagocytosis (6) and exposure to toxic oxygen radicals (20). Moreover, tissues that are affected by autoimmune inflammatory disorders also express the mammal counterpart of Hsp65 (14, 24). Expression of Hsp65 is also induced by exposure of THP-1 monocytic cells to IFN-γ and TNF-α, and these cytokines act synergistically in this respect (10). Our findings that Hsp65 by itself stimulates the release of TNF-α, IL-6, and RNI by peritoneal macrophages suggest that Hsp65, proinflammatory cytokines, and reactive metabolites may mutually reinforce their effects on the inflammatory reaction. These inflammatory changes induced by Hsp65 may contribute to protection against microbial pathogens at an early stage of infection before a species-specific or Hsp65 antigen-specific immune response is generated. The possible protective role of Hsp65 during infections is stressed by the data of another study showing that expression of high levels of Hsp65 in macrophages correlates with protection against infection with *T. gondii* (22). Also, the synergistic activity of small amounts of IFN-γ and Hsp65 as observed in the present study indicates a protective role for Hsp65 early during infection.

Our findings may provide new insight into the role of Hsp65 in autoimmune inflammatory disorders. The release of TNF-α and RNI following exposure to Hsp65 may provoke both tissue damage (4) and an upregulation of self Hsp65. We reported

recently that in intestinal lesions of Crohn's disease, self Hsp65 but not prokaryotic Hsp65 is abundantly expressed (24). Others have found that T lymphocytes directed at self Hsp65 are generated in draining lymph nodes of a sterile inflammation without previous exposure to exogenous Hsp65 (2). Self Hsp65 upregulated in inflammatory lesions may then be recognized by sensitized T lymphocytes raised against cross-reactive epitopes on Hsp65 homologs from unrelated species, e.g., microorganisms, or raised against self Hsp65. The latter event may lead to an Hsp65-mediated autoimmune inflammatory reaction.

The results of this study on the direct effect of Hsp65 on the functional characteristics of peritoneal macrophages provide further evidence for the hypothesis that Hsp65 may play a role in the initiation of the inflammatory process that accompanies infections with microbial pathogens and autoimmune disorders.

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