Heat Shock Protein 70 Is a Potential Virulence Factor in Murine *Toxoplasma* Infection Via Immunomodulation of Host NF-κB and Nitric Oxide

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Heat Shock Protein 70 Is a Potential Virulence Factor in Murine Toxoplasma Infection Via Immunomodulation of Host NF-κB and Nitric Oxide

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We propose that the 70-kDa heat shock protein (HSP70) protects virulent Toxoplasma gondii from the effects of the host by immunomodulation. This hypothesis was tested using quercetin and antisense oligonucleotides targeting the start codon of the virulent T. gondii HSP70 gene. Oligonucleotides were transiently transfected into two virulent (ME49, C) strains of T. gondii, significantly reducing HSP70 expression in treated parasites. Virulent parasites with reduced HSP70 expression displayed reduced proliferation in vivo, as measured by the number of tachyzoites present in spleens of infected mice. They also exhibited an enhanced rate of conversion from tachyzoites to bradyzoites in vitro. Our results implicate HSP70 as a means by which virulent strains of T. gondii evade host proinflammatory responses: when RAW 264.7 cells were exposed to parasites with reduced HSP70 expression, differential expression of inducible NO synthase (iNOS) and cell NO production were observed between infections with normal and HSP70-deficient T. gondii. iNOS message levels were significantly increased when host cells were infected with HSP70 reduced virulent tachyzoites and HSP70-related inhibition of iNOS transcription resulted in altered host NO production by virulent T. gondii infection. Virulent parasites expressing reduced levels of HSP70 initiated significantly more NF-κB activation in host splenocytes than infections with untreated parasites. Neither proliferative ability nor conversion from tachyzoites to bradyzoites was affected by lack of HSP70 in avirulent strains of T. gondii. Furthermore, avirulent T. gondii strains induced high levels of host iNOS expression and NO production, regardless of HSP70 expression in these parasites, and inhibition of HSP70 had no significant effects on translocation of NF-κB to the nucleus. Therefore, the 70-kDa parasite stress protein may be part of an important survival strategy by which virulent strains down-regulate host parasiticidal mechanisms. The Journal of Immunology, 2002, 169: 958–965.

Acute infection with a protozoan parasite such as Toxoplasma gondii typically initiates a cascade of cellular events in the immunocompetent host (1). For example, tachyzoite invasion of host cells induces IL-12 production in macrophages and dendritic cells (2, 3). In turn, IL-12 activates NK cells to produce IFN-γ, which stimulates macrophage production of TNF (4–6). These cytokines act synergistically to initiate signal transduction of the transcription factor NF-κB.

NF-κB performs an integral role in the regulation of immune response genes (7); it is an essential step in the innate immune response to pathogens (8). In their constitutive form, NF-κB dimers are sequestered in the cell cytoplasm by the inhibitor protein, IκB, which suppresses the nuclear localization signal of NF-κB (9). In response to the appropriate stimuli, IκB undergoes a process of phosphorylation, ubiquitination, and degradation, leading to the release and nuclear translocation of NF-κB, where it mediates transcription (10). NF-κB proteins activate gene transcription by binding to sites in the target promoters of a range of genes including those encoding cytokines, growth factors, and immunoreceptors. Of particular note is NF-κB’s effect on inducible NO synthase (iNOS)3, which produces the antimicrobial radical, NO (11), one of the key parasiticidal mechanisms used in intracellular protozoan infections.

In T. gondii infection, NF-κB (RelB) has been shown to be essential for the induction of innate NK and adaptive T cell responses that lead to the development of host resistance (12). However, a recent paper by Butcher et al. (13) has demonstrated the ability of this pathogen to invade cells without triggering proinflammatory responses. Indeed, T. gondii seems to be able to suppress NF-κB activity. The molecular basis for this suppression is not clear, but a potential explanation may rest with a 70-kDa heat shock protein (HSP70) of T. gondii associated with virulent parasite phenotypes of the parasite (14, 15). Thus, HSP70s are associated with protection against NO-induced cellular pathology and inhibition of NF-κB activity in a range of eukaryotic cells. Kim et al. (16) demonstrated that HSP70 antisense oligonucleotides abrogate NO-induced protection against TNF in rat hepatocytes, Bellmann et al. (17) showed that transfection of the human gene encoding HSP70 into rat insulinoma cells is protective against NO-induced cell lysis, and Feinstein et al. (18) found that stress-induced HSP70 reduces NF-κB nuclear uptake and subsequent downstream iNOS expression and activity in astroglial cells. Thus, in this study we tested the hypotheses that the HSP70 of T. gondii inhibits iNOS expression, NO production, and NF-κB activity, and that such effects are confined to virulent strains of the parasite.

3 Abbreviations used in this paper: iNOS, inducible NO synthase; HSP70, 70-kDa heat shock protein; PS, penicillin and streptomycin; HPRT, hypoxanthine phosphoribosyltransferase.
Materials and Methods

Cell culture

RAW 264.7 murine macrophages were a gift from Dr. G. Chaudry (Department of Pathology, University of Sydney, Sydney, New South Wales, Australia) in 1997. Since then, the cells have been passaged twice weekly and maintained in DMEM containing 5% FBS and 100 IU ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (PS).

Primary splenocytes were prepared from spleens of 6–8 wk old female BALB/c mice. Briefly, mice were killed by CO₂ overdose and cervical dislocation, and their spleens removed and retained in an excess of wash medium (DMEM and PS) and kept on ice. Under sterile conditions, the organs were poured onto a 70 μm nylon mesh (Falcon; BD Biosciences, Mountain View, CA) over a sterile 50-ml polypropylene tube (Falcon) and homogenized to produce a single-cell suspension. The homogenate was rinsed well with wash medium and centrifuged at 1500 rpm at 4°C for 5 min. The cell pellet obtained was resuspended in complete medium (DMEM, 10% FBS, PS). Viable cells were assessed by trypan blue dye exclusion test and counted using a Neubauer chamber.

Parasites and infection

Parasites used in these experiments were two virulent strains (RH, ENT) and two avirulent strains (ME49, C) of T. gondii. Parasites were cultured twice weekly in vitro in RAW 264.7 cells and grown in DMEM containing 5% FBS and PS.

Parasites were diluted in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, PBS) containing PS to a concentration of 1 × 10⁶ tachyzoites/ml. BALB/c mice were infected via i.p. injection with 1 × 10⁶ parasites. Mice were sacrificed at various intervals postinfection by CO₂ overdose and cervical dislocation and parasites harvested from the peritoneal cavity using a PBS wash.

Transient transfection

Expression of T. gondii HSP70 was reduced by transiently transfecting HSP70 antisense oligonucleotides into parasites, then treating parasites with quercetin, a bioflavonoid that specifically reduces HSP expression (19, 20). Phosphorothiate antisense oligonucleotides were designed to anneal to the T. gondii HSP70 ATG start codon. The antisense effect of the active oligonucleotide (Antia: 5′-CAC AGC AGG AGA GTG CGG CAT-3′, Tm = 71°C) was compared with a number of control oligonucleotides with varying degrees of target sequence homogeneity. Antib: four sequence mismatches, 5′-CAC AGC AGG AGA GTG CGA CAT-3′, Tm = 72°C; Antic: two sequence mismatches, 5′-CAC ATG AGG AGA GTG CGG CAT-3′, Tm = 71°C (underlined bases correspond to antisense sequence mismatches), and orientation; SenseA: active oligonucleotide in the sense orientation, ATG GCG GAC TCT CCT GCT GTG, Tm = 71°C.

Oligonucleotides were introduced into T. gondii tachyzoites via lipid-mediated transient transfection. Briefly, to each well of a 6-well plate (Costar, Cambridge, MA), 6 μl of lipid reagent DMRIE-C (Life Technologies, Rockville, MD) was added to 500 μl serum-reduced medium (Opti-Mem; Life Technologies). To this was added 500 μl Optiprem containing 4 μg DNA (the antisense effect was observed to be dependent on oligonucleotide concentration with 4 μg DNA proving optimal for transfection-induced HSP70 inhibition, as determined by repeated concentration optimization experiments). The total sample was mixed by gently swirling the plate, then incubated for 30 min at room temperature to allow formation of DNA-lipid complexes. A total of 2 × 10⁶ tachyzoites in 200 μl Optiprem was added and the solution mixed gently and incubated for 5 h at 37°C in 5% CO₂. Posttransfection, 2 ml of antibiotic-free medium was added to each sample and the parasites were left to recover overnight. Parasites were then resuspended in complete medium containing 50–100 μM quercetin (Sigma-Aldrich, St. Louis, MO) and incubated for 5 h at 37°C in 5% CO₂.

HSP70 assay

Quercetin-treated parasites were centrifuged and resuspended in complete medium before being subjected to heat stress (43 ± 0.5°C for 2 h). Parasites were assessed for cell viability (typically >90%) and counted, then collected and assayed for expression of HSP70 as described previously (22, 23). HSP70 expression was measured in a Bradford assay and 10 μg of sample was loaded onto an 8% SDS-PAGE gel (Gradipore, Sydney, Australia). Samples were transferred to a nitrocellulose membrane and HSP70 was detected by Western blot using an avian polyclonal chicken Ab targeting the GGMPGGG repeat at the 3′ end of T. gondii HSP70 (Chick29; Nerang Biotechnology, Melbourne, Australia). Chemiluminescent detection of signal (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) was performed and densitometric analysis (MultiAnalyst; Bio-Rad, Hercules, CA) of signal was used to quantify differences between samples.

Parasite burden

BALB/c mice were infected via s.c. injection with ~10⁶ tachyzoites. Spleens, livers, and lungs from infected mice were collected at 4 days postinfection to track parasite dissemination from the site of infection and assess parasite burdens. Each treatment group comprised four mice. Organs were sectioned using a scalpel blade and multiple imprints made by lightly pressing against a glass slide. Slides were then fixed and stained as described below. The mean and SE were calculated from the numbers of parasites in spleens from eight mice per condition.

Stage conversion

To promote tachyzoite-bradyzoite conversion, host RAW 264.7 cells (2.5 × 10⁴) were cultured in sterile 2-well Labtek chamber slides (Nalge Nunc International, Rochester, NY) in the presence of 100 ng/ml recombinant mouse IFN-γ (R&D Systems, Minneapolis, MN) and 10 μg/ml LPS (Sigma-Aldrich), then infected with ~10⁵ parasites. Slides were then fixed and stained as described below. The mean and SE were calculated from the numbers of parasites in spleens from eight mice per condition.

Immunocytochemical procedures

For immunocytochemistry analysis, imprint slides were washed in PBS before being fixed in 3% paraformaldehyde for 10 min. Samples were incubated in methanol at 4°C for 10 min then blocked with 2.5% goat serum in 5% low fat milk in PBS for 30 min. For ease of parasite counting in dissemination experiments, slides were stained with anti-T. gondii polyclonal Ab in PBS with 2.5% goat serum for 45 min, followed by anti-goat IgG TRITC-conjugated secondary Ab (Sigma-Aldrich), both at a dilution of 1/50. For histochemical detection of tachyzoites in imprints, slides were fixed in methanol for 20 min, then stained in freshly diluted May-Grünwald stain for 5 min. Excess stain was removed then slides immersed in fresh Giemsstain for 10 min followed by three rapid washes in buffered dilH₂O. Slides were allowed to dry before mounting with a coverslip. Fluorescent staining tachyzoites were counted from 20 fields of view per slide for four mice in each of two experiments.

Differentiation of T. gondii parasites was assessed by expression of tachyzoite- and bradyzoite-specific markers SAG1 and BAG1, detected using DG52 anti-SAG1 polyclonal Ab and 7ES anti-BAG1 mAb at dilutions of 1/100 (kindly provided by Prof. J. Boothroyd, Stanford University, Stanford, CA, and Prof. U. Gross, Georg-August-University, Goettingen, Germany, respectively). Parasites were subsequently reacted with anti-mouse IgG FITC conjugate secondary Ab (Sigma-Aldrich) at a dilution of 1/64. As a control for cross-reactivity, some samples were stained with secondary Ab only. The percentage of parasites undergoing stage conversion, as assessed by BAG1 and SAG1 expression, was evaluated using fluorescent microscopy. Twenty fields of view per chamber were counted in duplicate in three separate experiments.

iNOS analysis

RAW 264.7 cells (2.5 × 10⁴) were cultured in sterile 75 cm² flasks (Falcon) in the presence of 100 ng/ml recombinant mouse IFN-γ (R&D Systems) and 10 μg/ml LPS (Sigma-Aldrich), then infected with 1 × 10⁶ tachyzoites. After a 3-h incubation, infected cultures were washed in PBS and the cells washed in cold PBS before being harvested for RNA isolation. TRIZol reagent (Life Technologies) was added directly to culture medium before being subjected to RNA extraction with TRIzol reagent according to the manufacturer’s instructions. The resultant cDNA was amplified for each sample in a multiplex PCR.

mRNA was isolated from total RNA using Dynabeads (Dynal Biotech, Hercules, CA) of signal was used to quantify differences between samples. The Journal of Immunology

The percentage of parasites undergoing stage conversion, as assessed by BAG1 and SAG1 expression, was evaluated using fluorescent microscopy. Twenty fields of view per chamber were counted in duplicate in three separate experiments.

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Nitrite, the stable byproduct of NO, was analyzed in the culture supernatant of macrophages primed with 100 ng/ml IFN-γ and 10 μg/ml LPS, then infected with 1 × 10⁷ tachyzoites, using the Griess colorimetric reaction (24). Briefly, supernatant (100 μl) was reacted in a mixture of 50 μl 1% sulfanilamide (Sigma-Aldrich) and 50 μl 0.1% N-(1-naphthyl)ethylene diamine dihydrochloride (Sigma-Aldrich) in 3% phosphoric acid at room temperature. The absorbance was read after 5 min in a microtiter plate reader at 540 nm in reference to a standard nitrite quantitative curve.

**Statistical analysis**

Statistical significance was assessed by the Student’s t test and/or the one-way ANOVA with Tukey’s multiple comparison test, where indicated, using GraphPad Prism version 3.0 for Windows software (GraphPad, San Diego, CA). Comparisons were considered significant at p ≤ 0.05.

**Results**

**Effect of antisense oligonucleotides and quercetin treatment on HSP70 expression in T. gondii**

Optimal HSP70 inhibition was achieved using a combination of antisense and quercetin treatment. Parasites treated with quercetin alone reduced HSP70 expression by 50% compared with untreated parasites, while treatment with the active antisense oligonucleotide AntiA alone reduced stress protein expression by 55% (one-way ANOVA with Tukey’s multiple comparison test; Fig. 1). Transfection of *T. gondii* tachyzoites with AntiA antisense oligonucleotides and subsequent treatment with quercetin reduced HSP70 expression by 77% (significant at p < 0.05; one-way ANOVA with Tukey’s multiple comparison test) after samples were stressed in vitro, compared with values generated in control samples, and by 69% compared with the SenseA oligonucleotides (Fig. 1); the effect was concentration-dependent (data not shown). Parasites were assessed for cell viability (typically >90%) and counted, then collected and assayed for expression of HSP70 over a 96-h period as described previously (22, 23). HSP70 expression was reduced in parasites for up to 72 h posttreatment both in vitro and in vivo (by 77 and 87%, respectively), but expression of the stress protein returned to control levels after this period. Control oligonucleotides showed increasing antisense effect with increases in sequence homogeneity corresponding to the complementary region of HSP70 targeted by the active antisense oligonucleotide. For example, the SenseA oligonucleotides inhibited HSP70 expression by 27% compared with untreated tachyzoites, but this was not statistically significant. Likewise, the 16% reduction in HSP70 expression seen in the presence of the AntiB antisense oligonucleotides (four sequence mismatches) was not significant, but treatment with AntiC (two sequence mismatches) achieved a 50% reduction in parasite stress protein expression, which was a significant reduction (p < 0.05; one-way ANOVA with Tukey’s Multiple Comparison test).

HSP70 expression was also reduced by AntiA antisense oligonucleotide treatment in parasites recovered from the peritoneal cavity of mice (Fig. 2). In parasite samples pooled from four mice per condition, expression of HSP70 by the virulent RH and ENT

**FIGURE 1.** HSP70 expression in *T. gondii* in response to heat shock in vitro. Tachyzoites of RH strain *T. gondii* were treated with different oligonucleotides (SenseA, AntiA, B, or C) and/or quercetin (Q) before being subjected to heat stress in vitro to induce HSP70 expression. HSP70 expression was assessed by Western blot and determination of the densitometric values for the HSP70 band. Densitometric values for each sample were adjusted for background signal. Results are the mean ± SE of densitometric values obtained from three transfection experiments and assessed using one-way ANOVA with Tukey’s multiple comparison test. At p < 0.05, each of the groups RH + 50 μM quercetin, RH + AntiA, RH + AntiC, and RH + AntiA + 50 μM quercetin, was significantly different from the RH group. Additionally, the RH + AntiA + 50 μM quercetin was significantly different from the RH + 50 μM quercetin and the RH + AntiA groups.
strains was reduced by 87 and 78%, respectively, whereas expression of HSP70 in the avirulent strains, ME49 and C, was reduced by 50% in both cases.

**Parasite burden in hosts infected with strains with reduced parasite HSP70 expression**

Tachyzoites were rarely detected in the liver and lungs of infected mice. However, spleens from mice infected with virulent RH strain parasites contained significantly more (*p* < 0.001; Student’s *t* test) parasites than did mice infected with virulent parasites treated to reduce HSP70 expression. In fact, for virulent infections, a 45% (RH strain) and 25% (ENT strain) reduction in splenic parasite burden was observed after HSP70 inhibition in these parasites (Fig. 3). Avirulent strains, which were detected in much lower numbers in the spleen compared with virulent strains, showed no significant difference between normal and HSP70-treated parasites (Fig. 3).

**HSP70 and parasite differentiation**

Parasites cultivated in the presence of activated macrophage host cells were positive for BAG1 by 48 h postinoculation; however, detection of bradyzoite-specific Ags varied between strains and treatment groups. In virulent strains, detection of bradyzoite-specific Ags was always a low percentage of the total population (<5%) compared with expression of the tachyzoite-specific SAG1 molecule; however, virulent strains with reduced HSP70 expression showed significantly increased expression of the BAG1 bradyzoite marker compared with untreated virulent parasites (*p* < 0.05; one-way ANOVA with Tukey’s multiple comparison test). BAG1 expression in RH strain was increased by 13–62% when parasites were treated for HSP70 expression, while in the other virulent strain examined, ENT, BAG1 expression rose by 18–75% upon HSP70 inhibition (Fig. 4).

In contrast, avirulent strains (ME49, C) showed uniformly high BAG1 expression after 48 h of culture with activated immune cells (14–22% of the total population). Although not significant, a slight decrease in bradyzoite Ag expression and a corresponding increase in tachyzoite-specific SAG1 were observed in avirulent strains treated to reduce HSP70 expression (Fig. 4).

**Effect of *T. gondii* HSP70 on iNOS activity**

The combination of IFN-γ and LPS stimulated expression of iNOS in RAW 264.7 cells (Fig. 5). Infection of these cells with tachyzoites of an avirulent strain (ME49) of *T. gondii* caused a 45% increase in expression of this protein (significant at *p* < 0.001; one-way ANOVA with Tukey’s multiple comparison test). Treatment with the AntiA antisense oligonucleotides did not affect the ability of the avirulent tachyzoites to induce iNOS expression. In contrast, infection of IFN-γ/LPS-primed RAW 264.7 cells with virulent tachyzoites (RH strain) dramatically reduced expression of iNOS (by 95%, *p* < 0.001; one-way ANOVA with Tukey’s multiple comparison test). Treatment of RH tachyzoites with the AntiA antisense oligonucleotides significantly (*p* < 0.01; one-way ANOVA with Tukey’s multiple comparison test) reversed this effect (expression of iNOS was 4-fold higher in the presence of transfected parasites), but the expression of this enzyme in the presence of the transfected parasites was still substantially (79%) less than that of the uninfected RAW 264.7 cells (*p* < 0.001; one-way ANOVA with Tukey’s multiple comparison test).

Analysis of the stable byproduct (NaNO₂) of NO in culture supernatant of infected RAW 264.7 cells, primed with rIFN-γ and LPS, confirmed the affect of the AntiA antisense oligonucleotides on iNOS expression. Thus, IFN-γ and LPS stimulated NO production in RAW 264.7 cells and this was further enhanced (by 45%, significant at *p* < 0.01; one-way ANOVA with Tukey’s multiple comparison test) by infection with avirulent ME49 tachyzoites (Fig. 6).
when compared with avirulent infections; 29% of RH strain-exposed cells showed only cytoplasmic NF-κB expression. In the absence of inducing signals, 92% of uninfected splenocytes was negative for the presence of nuclear p65. This translocation was only observed in 44% of ME49 strain-exposed cells and in 40% of C strain-exposed cells. However, nuclear translocation of NF-κB was reduced in cells exposed to RH strain and ENT strain tachyzoites when compared with avirulent infections; 29% of RH strain-exposed cells and 34% of ENT strain-exposed cells were positive for nuclear p65. This effect was significantly reversed (p < 0.05; one-way ANOVA with Tukey’s multiple comparison test) by treatment of the virulent parasites with AntiA antisense oligonucleotides. Thus, transfection of the avirulent parasites with AntiA antisense oligonucleotides targeting the translation initiation codon of HSP70 minimally reduced HSP70 expression in vitro and in vivo. The inhibition of HSP70 expression was more obvious in virulent strains of T. gondii than avirulent strains.

Effect of T. gondii HSP70 on nuclear translocation of NF-κB
In the absence of inducing signals, 92% of uninfected splenocytes showed only cytoplasmic NF-κB expression (Fig. 7). Con A, a known stimulator of NF-κB translocation to the nucleus, induced nuclear uptake of p65 in 40% of cells after 3 h. Infection with tachyzoites of avirulent strains of T. gondii also stimulated substantial translocation of NF-κB to the nucleus; >65% of ME49 strain-exposed cells and >50% of C strain-exposed cells were positive for the presence of nuclear p65. This translocation was only slightly affected by treatment of the parasites with AntiA antisense oligonucleotides; transfection of NF-κB to the nucleus occurred in 44% of ME49 strain-exposed cells and in 40% of C strain-exposed cells. However, nuclear translocation of NF-κB was reduced in cells exposed to RH strain and ENT strain tachyzoites when compared with avirulent infections; 29% of RH strain-exposed cells and 34% of ENT strain-exposed cells were positive for nuclear p65. This effect was significantly reversed (p < 0.05; one-way ANOVA with Tukey’s multiple comparison test) by treatment of the virulent parasites with AntiA antisense oligonucleotides. Thus, transfection of the avirulent parasites with AntiA antisense oligonucleotides reversed HSP70 expression.
during acute infection by avirulent strains is unknown. However, our results with HSP70 inhibition in virulent strains implicate HSP70 expression as a component of the virulence phenotype, somehow facilitating ongoing replication of the proliferative tachyzoite form in the face of cytostasis and reduced replication in avirulent and HSP70-reduced virulent strains. This hypothesis is further supported by our observations of increased differentiation signaling, in terms of BAG1 expression, in virulent parasites treated to reduce HSP70 expression. Thus, inhibition of HSP70 expression in virulent strains of T. gondii (RH and ENT) had profound effects on strain propensity to differentiate following infection, which would commence, resulting in dissolution of the NF-κB complex. Nuclear uptake of the transcription factor then commences, but at this time, HSP70 levels are still low, so initial nuclear uptake is not yet fully elucidated. In cases involving HSP70-mediated inhibition of NF-κB, Feinstein et al. (18) proposed that stress proteins physically interact with IκB. The association of inhibitory IκB with NF-κB subunits occurs via interaction with IκB ankyrin domains with nuclear localization sites present in the p50 and p65 proteins. Nuclear localization site homologs in human HSP70 have been described, thus raising the potential for HSP70 interaction with ankyrin domains present in IκB. This activity could result in prevention of IκB phosphorylation and subsequent dissociation of NF-κB. However, this explanation seems unlikely to account for the effect of T. gondii HSP70, because it has recently been shown that although tachyzoites do invade macrophages without activating NF-κB, they trigger normal phosphorylation-dependent degradation of IκB, thus indicating that the block on NF-κB translocation occurs downstream of these events (13).

Other possibilities are that HSP70, which is capable of migrating to the nucleus upon induction of the stress response, impedes NF-κB nuclear translocation by competing for access to the same nuclear pore complexes through which NF-κB must be transported to the nucleus (18). HSP70 could potentially inhibit NF-κB activation by direct interaction with one or more of the NF-κB constituents. Upon dissolution of the NF-κB/IκB complex after signal-induced activation, NF-κB is left in a temporarily unstable state that may imitate that of a denatured protein. HSP70 could, in a move which adheres to its functional capacity as a molecular chaperone, bind to and therefore, prevent the nuclear translocation of NF-κB as it dissociates from IκB. Thus, the subcellular localization of the HSP70 protein and the timing of its production may be important to promoting suppressive effects. Feinstein et al. (18) hypothesized that following activation of the signals necessary to induce NF-κB nuclear translocation and iNOS induction, a period of time is required such that HSP70 is present concurrently with the HSP70-sensitive step in this cascade of events. Thus, HSP70 expression begins within 30 min and continues to accumulate for the next several hours. During this time, translocation of host NF-κB would commence, resulting in dissolution of the NF-κB/IκB complex. Nuclear uptake of the transcription factor then commences, but at this time, HSP70 levels are still low, so initial nuclear uptake is probably not impeded. However, within the next phase of activation, HSP70 levels are sufficient to reduce NF-κB uptake to the nucleus. This timing fits very nicely with the timings of suppression of immune activities observed by Butcher et al. (13). Furthermore, our observation that the inhibition of NF-κB translocation was not complete (an observation also made by Butcher et al., Ref. 13) fits the above sequence of events. Thus, this is a plausible explanation for the immunomodulatory effects of T. gondii HSP70, but it begs the question of how the parasite HSP70 gains access to the host cytoplasm and nucleus. It will be interesting to see whether HSP70 is released during, or shortly after, host cell invasion by virulent tachyzoites, along with several other proteins that could conceivably interfere with host signaling pathways (13). We do have preliminary evidence that HSP70 is
indeed secreted by *T. gondii*; analysis of precipitated proteins separated by two-dimensional electrophoresis and probed with a parasite specific anti-HSP70 Ab detected parasite HSP70 in the culture supernatant at 4 h postinfection (data not shown). Furthermore, a number of examples from other parasites have described extracellular HSP70; for example, a 72-kDa *Plasmodium falciparum* HSP related to the 78-kDa glucose-regulated stress protein of mammals called Pgrp (27) was detected in sera of people exposed to this parasite (28), and is expressed on the surface of infected hepatocytes (29).

There are several ways by which HSP70 might arrive at the cell surface and from there, move to the extracellular space. For example, in *P. falciparum* it is hypothesized that before merozoite formation, HSP70 associates with incipient membranes destined to constitute the merozoite surface, and within this molecular complex, locates to the cell surface (30). Additionally, HSP70 may be cytoplasmic during early parasite development but adheres to the surface of parasites after differentiation, or during host cell lysis (30). Alternatively, parasite HSP70 may become extracellular by passive anchoring to the cell surface of proteins released from dead or dying cells; for example, promonocytic cells are able to interact with and internalize HSP70 members (31). The exact nature of HSP70 release from the *T. gondii* remains to be elucidated; however, detection of this protein in the culture medium of infected cells suggests that *T. gondii* HSP70 is at least accessible to the host machinery, and therefore, in the position to exert the immunomodulatory effects observed in this study. This mechanism could help to explain why the inhibition of iNOS and NO production by virulent *T. gondii* in vitro was so efficient (95% reduction in iNOS expression in 2.5 × 10^5 activated RAW cells exposed to only 10^5 tachyzoites).

An additional issue raised by our results is why HSP70 from virulent *T. gondii* tachyzoites has such dramatic effects, whereas the same protein in avirulent strains of the parasite fails to affect host cell responsiveness or parasite differentiation? Molecular genetic studies show that the HSP70 gene is single copy in both virulent and avirulent strains of *T. gondii* (14). However, sequence analysis has revealed that the genes encoding HSP70 in virulent and avirulent strains are identical at the amino acid level, with the exception of the number of seven residue repeat units (GGMPGGM) at the 3′-end of the gene. In avirulent strains, five copies of this repeat are detected, while virulent parasites have only four (15). As the only difference between strains in terms of genetic structure, the deletion of this unit may have important implications for the synthesis and stability of this protein, which in turn may affect its efficacy in terms of immunomodulation and stage conversion. It is possible that HSP70, a molecule whose three-dimensional configuration is still being investigated, selectively presents its hypervariable C termini to the host immune system, which in turn may result in increased infectivity while concurrently acting as a trigger for components which protect the parasite from host defense mechanisms such as NO. Whether this accounts for the differential HSP70-directed response of virulent and avirulent strains to the host immune response and induction of parasite differentiation remains to be seen.

A potential limitation of the protocols used in this study to inhibit HSP70 expression by *T. gondii* (i.e., the combination of transient transfection with antisense oligonucleotides and quercetin) is that quercetin and other related flavonoids are known to induce multiple changes in the cells exposed to them (32–35). The complex nature of the relationship between flavonoid compounds such as quercetin, heat shock proteins, and the immune response obviously requires further characterization, but it should be emphasized that, in this study, only parasites were treated with quercetin; the host cells that were used to examine the effects of parasite HSP70 on various parameters of the immune response were not exposed directly to quercetin at any time. Thus, the effects we have observed are highly likely to be the result of inhibition of parasite HSP70 by the combined action of the antisense oligonucleotides and quercetin. However, it is remotely conceivable that quercetin, independent of its effects on parasite HSP70, may have contributed to the effects on key host immune effector events and the promotion of bradyzoite formation that were observed in this study. Experiments with stable HSP70-knockout parasites, where transcription levels as well as expression can be confirmed, may definitively resolve this issue.

Regardless of the HSP70 sensitive step in the cascade of events leading to inhibition of NF-κB activation and iNOS transcription in virulent *T. gondii*, HSP70 does appear to play a role in the ability of virulent parasites to modulate immune response factors. Our results implicate HSP70 as a means by which virulent strains of *Toxoplasma* evade proinflammatory responses. The fact that the effects of HSP70 are more apparent in virulent than avirulent strains is consistent with observations of unrestrained virulent strain asexual replication compared with the apparently enforced encystations and subsequent development of quiescent parasitic forms observed in avirulent strain infection. We hypothesize that HSP70 may have different functions in avirulent and virulent *T. gondii* parasites: in avirulent parasites, which readily undergo cyst formation, HSP70 may be part of the machinery involved in stage conversion, facilitating an essential evasion strategy of the parasite. The role of HSP70 in virulent parasites, which do not readily differentiate into cyst-dwelling bradyzoites, could represent an alternative mechanism by which the parasite avoids the host immune response. This stress protein may be part of an important survival strategy by which virulent strains down-regulate activation of NO production, thereby removing one of the principal parasiticidial mechanisms from the host-parasite equation.

### References