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Immunogenicity of *Toxoplasma gondii* Heat Shock Protein 70

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ABSTRACT

Heat shock proteins (HSPs) are a group of proteins involved in maintaining cell homeostasis. Their synthesis is increased dramatically in response to stimuli such as temperature shifts, changes in concentrations of glucose and calcium and also in response to immune factors. HSP70 has been found to be strongly expressed in virulent strains of *Toxoplasma gondii* taken from immunocompetent mice but poorly expressed in avirulent strains taken from immunocompromised mice or virulent and avirulent strains cultivated in vitro. Hence, *T. gondii* HSP70 may help protect parasites against the host's immune response, allowing the virulent strains to persist as tachyzoites without the enforced encystation found in avirulent strains. This makes *T. gondii* HSP70 an attractive subunit candidate. To investigate the immunogenicity of this protein it was affinity purified from tachyzoites of the virulent RH strain and injected into BALB/c mice along with Freund's Complete Adjuvant (FCA). A booster shot with Freund's Incomplete Adjuvant (FIA) was given 4 weeks after the initial injection and blood was collected at 8 weeks. The RH strain HSP70 DNA was cloned as a GST fusion protein, expressed and also injected into BALB/c mice to see if the immune response generated by the recombinant protein matched that of the native protein. Antibody response was measured by ELISAs to detect IgG1, IgG2a, IgG2b and IgG3. An elevation of IgG1 antibodies, but not other IgG subsets, was observed in the mice immunised with native or recombinant HSP70. This suggests that the immune response seen with the recombinant protein is similar to that seen with the native protein. However, the response to either protein was weak, suggesting that

the *T. gondii* HSP70 may not be a particularly good immunogen.

INTRODUCTION

Heat shock protein 70 (HSP70) is a member of a group of heat shock or stress-induced proteins involved in maintaining cell homeostasis. Along with the other HSP families, it has a number of functions including: (1) binding to steroid receptors thereby preventing their binding with nuclear DNA until the steroid is also bound; (2) facilitation of transport, folding, assembly, biosynthesis and secretion of proteins; and (3) binding to unfolded, denatured or abnormal proteins and acting as a "molecular chaperone", allowing the proteins to refold correctly. Synthesis increases dramatically in response to stimuli such as temperature shifts, changes in concentrations of glucose and calcium and also in response to immune factors such as free oxygen radicals and cytokines (reviewed by Kaufmann, 1990).

HSP70 has been observed frequently in parasites and, despite a high degree of homology with vertebrate HSP70, has proved to be highly immunogenic for many helminth and protozoan parasites. This antigenicity is very specific. For example, antibodies induced to HSP70s during *Schistosoma japonicum* and *Schistosoma mansoni* infections do not cross react (Hedstrom et al. 1988) and sera from patients with *Plasmodium vivax* malaria do not react with peptides unique to *Plasmodium falciparum* HSP70 (Kumar et al. 1990). The most antigenic regions of parasite HSP70 are located in the variable carboxy-terminal region of the protein and not in the highly conserved amino-terminal region (Hedstrom, et al. 1988; Wallace et al. 1992) and, so, there has been interest in parasite HSPs as constituents of anti-parasite subunit vaccines (Sharma 1992).

HSP70 has been found to be strongly expressed in virulent strains of *Toxoplasma gondii* taken from immunocompetent mice but poorly expressed in avirulent strains. Furthermore, virulent strains taken from immunocompromised mice or cultivated in vitro do not express HSP70 (Lyons & Johnson 1995). This implies that in *T. gondii,* HSP70 expression is induced as a response to stresses imposed by the host's immune response. HSP70 may help protect parasites against the host's immune response, allowing virulent strains to persist as tachyzoites without the enforced encystation found in avirulent strains. Such an important function makes the *T. gondii* HSP70 an attractive subunit vaccine candidate. The aim of this study was to (1) purify native HSP70 from virulent RH *T. gondii;* (2) clone and express a recombinant glutathione S-transferase (GST) fusion protein version of the RH HSP70; and (3) investigate the immunogenicity of *T. gondii* RH strain HSP70 and to determine the type of immune response activated by this HSP in order to indicate its usefulness as a vaccine candidate.

MATERIALS AND METHODS

Animals

Female BALB/c mice of 6-8 weeks of age were used in the experiment. They were housed in the Gore Hill Research Laboratories on a 12 hrs light/12 hrs dark cycle at 21°C. Food and water were freely available.

Parasites

The virulent RH strain of *T. gondii* was used in these experiments. It has been maintained by intra-peritoneal (i.p.) passage in mice since isolation. BALB/c mice were infected with 10^5 tachyzoites i.p. then killed 4 days post infection by cervical dislocation under anaesthetic. The peritoneal fluids were harvested after injection of 1 ml PBS into the peritoneal cavity. Tachyzoites and red blood cells were pelleted by centrifugation (2,000 rpm for 10 min at 4°C) and resuspended in 10 ml lysis buffer (150mM NH_4Cl, 0.1M TrisHCl pH 7.4) for 10 min at room temperature. Tachyzoites and host cells were counted by direct microscopy using a Neubauer Counting Chamber. Parasites were pelleted by centrifugation (2,000 rpm for 10 min at 4°C) and pellets were stored at -70°C until used.

Antibodies

Antibodies used in Western Blotting were: (1) a rabbit derived polyclonal antiserum made against a cDNA clone (Ag63) which contains 939 bp of an HSP70-encoding gene of *Plasmodium falciparum* (Bianco et al. 1986). This serum was kindly provided by Dr. Robin Anders (WEHI) and has been used previously to detect the *T. gondii* HSP70 (Lyons & Johnson 1995); (2) a commercial murine monoclonal antibody (MA3-007) specific for an epitope located between amino acids 122 and 264 of human HSP70 thought to be the ATP binding site (Affinity Bioreagents); and (3) a commercial avian polyclonal antibody (Nerang Biotechnology) made against a [GGMPGGM]3 repeat peptide conjugated to Diptheria toxin (Auspep). This repeat sequence is located at the 3' end of the *T. gondii* HSP70 gene (Lyons & Johnson 1998). The antibodies used in the ELISAs (anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG3) were obtained from The Binding Site.

Purification of HSP70

Native HSP70 was purified from tachyzoite pellets using a single step method (Nandan 1994). Tachyzoites were reduced to protein extracts by resuspension of the pellets in lysis solution (20mM Tris pH7.5, 0.15M NaCl, 1 % Triton X-100, 1 mM ethylenediaminetetraacetic acid, 1 mM benzamidine HCl and 2 mM dithiothreitol) and disruption by sonication at 50W/20KHz) for 4 x 30 sec.

The resulting preparation was centrifuged at 2,000 rpm for 10 min and the supernatant retained.

Adenosine triphosphate (ATP) agarose (Sigma) was swollen in 20 mM Tris pH7.5, 0.15 M NaCl and added to the supernatant and the mixture rotated on a wheel for 2 hrs at room temperature. The ATP agarose was collected by centrifugation at 500*g* for 5 min and washed 3 times with 20 mM Tris pH 7.5, 0.5 M NaCl, 1% Triton X-100 then once with 5 mM HEPES pH 7.5. Native protein was eluted by mixing the ATP agarose with 5 mM HEPES pH 7.5, 1 mM $MgCl_2$, 3 mM ATP for 2 x 15 min at room temperature and collecting the eluate by centrifugation at 500*g* for 10 min. The protein concentration was determined using the Bradford dye-binding assay (BioRad).

Cloning of RH HSP70

Genomic DNA was isolated from tachyzoite pollets by standard sodium dodecyl sulphate (SDS)/ proteinase K lysis. For amplification of the entire HSP70 gene, forward primer HSPBF (5'AAG AGG ATC CAT GGC GGA CTC TCC TGC TG 3') and reverse primer HSPBR (5'CGT TTG GAT CCT TAA TCA ACT TCC TCC ACG3') were designed based upon the RH HSP70 gene sequence (Lyons & Johnson 1998) and incorporating a BamH1 site (underlined) for ease of cloning. The reaction mixtures [67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton-X100, 0.2 mg/ml gelatin, 2 mM $MgCl_2$, 200 μM each dNTP, 1 unit Taq (Fisher-Biotec), 50 pmol each primer and 100 ng genomic DNA in 100 μl final volume] underwent 30 cycles of denaturing at 93°C for 1 min, annealing at 61°C for 2 min and extension at 72°C for 2.5 min with the initial denaturation for 3 min and the final extension for 5 min. A single product of the correct size (2,028 bp) was amplified under these conditions. It was digested with BamH1 and ligated into pGEX-1λt. Positive clones were identified and expression of fusion protein induced with 0.1 mM isopropyl-β-D-Thiogalactopyranoside (IPTG). Fusion protein was affinity purified using glutathione agarose following the manufacturer's instructions (Amersham).

SDS-PAGE and Western Blotting

Purified native and recombinant HSP70 was analysed by SDS-PAGE (Laemmli 1970) using 8% Tris glycine gels (Gradipore) under denaturing conditions. After electrophoresis, protein was transferred to PVDF membrane (Millipore) as described by Towbin et al. (1978). The membrane was incubated with phosphate-buffered saline (PBS) with Tween 20 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.3% Tween 20) containing 5% nonfat dry milk for 30 min to block the membrane. After washing with PBS/Tween 20,

the membranes were incubated with primary antibody (polyclonal serum Ag63, polyclonal chicken anti-repeat or MA3-007) at a dilution of 1/1000 for 1 hr. After washing with PBS/Tween 20 the membranes were then incubated with goat anti-rabbit-, rabbit anti-chicken- or goat anti-mouse-horseradish peroxidase conjugated secondary antibody at a 1/1,000 dilution for 1 hr. The membranes were subsequently incubated with enhanced chemiluminescence (ECL) reagents (Amersham) for 1 min and exposed on film for development.

Immunisation with native and recombinant HSP70 of *T. gondii*

Mice were divided into groups and injected with 10μg of native protein emulsified in Freund's Complete Adjuvant (FCA), 10μg of recombinant protein in FCA or 10μg of a crude extract of *T. gondii* in FCA. Controls included mice injected with PBS/FCA and mice injected with GST/FCA or not injected at all. Booster shots of all proteins were administered at 4 weeks using Freund's Incomplete Adjuvant (FIA). Blood was collected via cardiac puncture under NO_2/O_2/halothane at 8 weeks.

Detection of anti-HSP70 antibodies

Sera from immunised mice were assayed for IgG1-, IgG2a-, IgG2b- and IgG3-reactivity to *T. gondii* HSP70 using a modification to a previously described enzyme-linked immunosorbent assay (ELISA) (Smith et al. 1997): ELISA plates were coated with 0.1 μg/well of native *T. gondii* HSP70; sera were assayed at a dilution of 1:100 ; anti-IgG subset/biotin conjugates were used at a dilution of 1:6,000 (determined in a checkerboard assay); alkaline phosphatase-conjugated extravidin (Sigma) was used at a dilution of 1:5,000. Mean$\pm$SE were determined for each group and significant differences calculated using Mann-Whitney non-parametric statistics.

RESULTS AND DISCUSSION

The native protein purified by the single-step method described was analysed using SDS-PAGE and Western blotting. It was found to be a single band with an apparent molecular weight of about 77 kDa and was recognised by the polyclonal rabbit antiserum to *P. falciparum* HSP70, the polyclonal chicken IgG raised against the GGMPGGM repeat and the commercial monoclonal to the ATP binding site of human HSP70 (Fig. 1) indicating that it is HSP70 from *T. gondii* RH strain. It corresponded to a protein observed in a crude protein extract of tachyzoites of *T. gondii* RH strain which was recognised by all antibodies used (Fig. 1) and which was no longer found in the crude extract following purification (data not shown).

SDS-PAGE and Western blotting analysis of the expressed fusion protein recovered from glutathione agarose showed a protein of approximately 96 kDa i.e. GST plus HSP70 (Fig. 1). This protein was recognised by the antiserum to *P. falciparum* HSP70 and the chicken antibody to the [GGMPGGM]3 repeat of the *T. gondii* HSP70; both of these antibody preparations recognised the native HSP70 showing that the 96 kDa fusion protein is the product of a clone of *T. gondii* RH strain HSP70 DNA. Two smaller proteins present in the preparation were also recognised by the polyclonal rabbit antiserum. These seem to be truncated versions of the fusion protein as the chicken IgG to the repeat located at the 3' end of the protein only recognises the full length product. The monoclonal to the ATP binding site does not recognise the recombinant version of the protein at all suggesting that it is not folded properly. However, the response seen with the other two antibodies suggests that this does not affect it's immunogenicity. The murine monoclonal antibody also recognised some lower molecular weight proteins in the *T. gondii* RH strain crude protein extract which appear to be other ATP binding proteins. None of the antibodies used recognised the peptide specific to human inducible HSP70 (Fig. 1) suggesting that the HSP70 isolated from RH tachyzoites and the cloned version are not related to the inducible form of human HSP70.

An elevated IgG1 response was seen in mice injected with native HSP70 ($p<0.002$) and in mice injected with recombinant HSP70 ($p<0.002$, Fig. 2a). No significant IgG1 response was observed in mice injected with PBS, crude *T. gondii* RH strain extract or GST or in untreated mice. No significant IgG2b or IgG3 antibody responses in any group was observed (Figs. 2b, 2c and 2d). Overall, the absorbances at 405 nm were quite low indicating a fairly weak response.

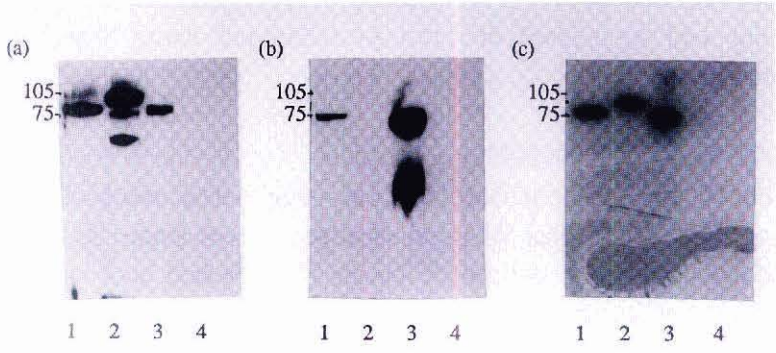

Figure 1. Immunoblot analysis of crude and purified protein preparations using (a) polyclonal antiserum to Ag63; (b) monoclonal antibody MA3-007 and; (c) chicken IgG raised to the [GGMPGGM]3 repeat peptide conjugated to Diptheria toxin. Lane 1 in each blot contains 3 μg of purified native RH strain HSP70, lane 2 contains 12 μg of recombinant RH strain HSP70, lane 3 contains 10 μg of a crude protein extract of *T. gondii* RH strain and lane 4 contains 100 ng of a peptide specific for human inducible HSP70.

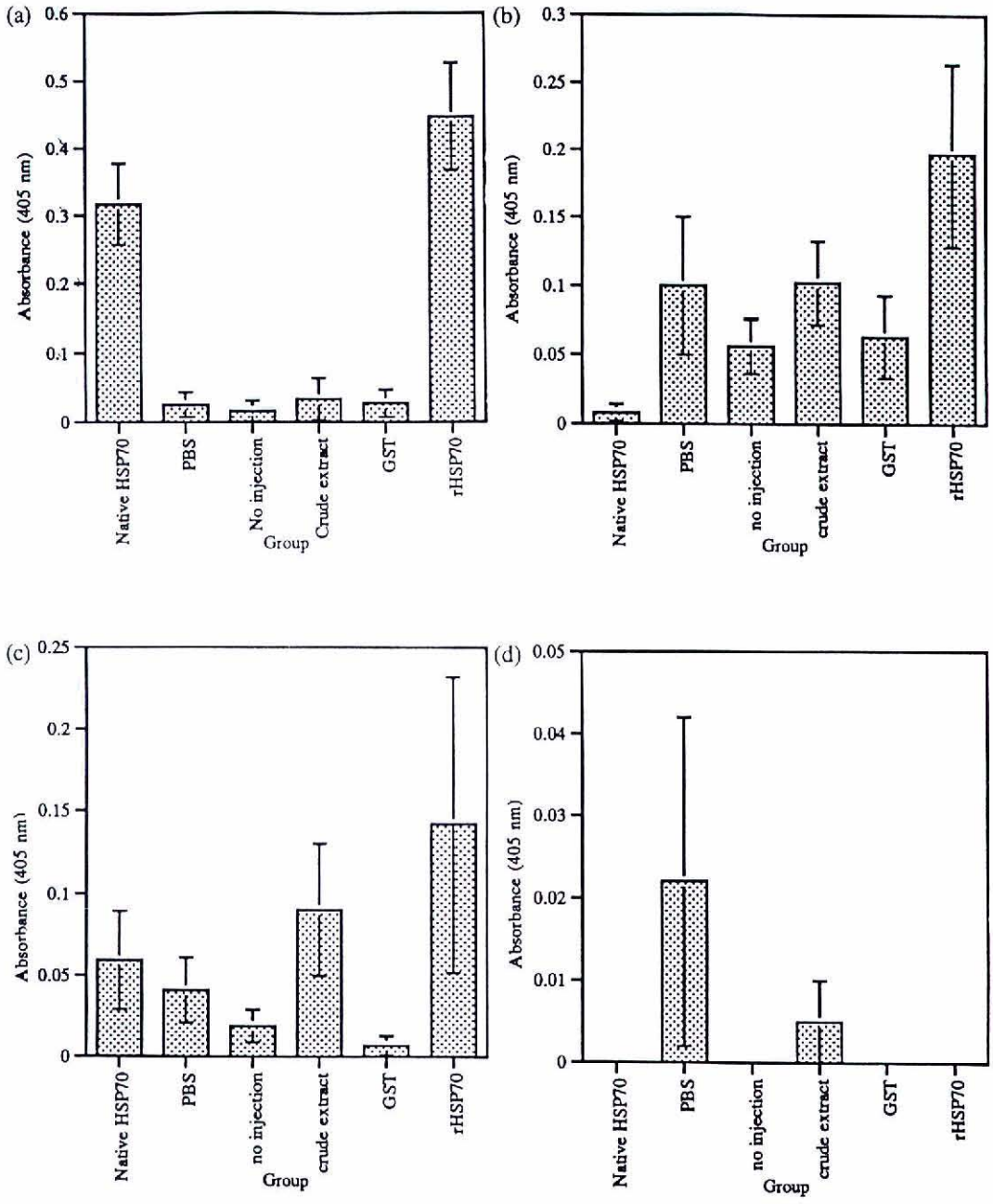

Figure 2. Antibody responses stimulated by injection of BALB/c mice with 10 μg of native *T. gondii* RH strain HSP70, 10 μg of recombinant RH strain HSP70 or 10 μg of a crude extract of *T. gondii* RH tachyzoites. Serum was assayed by ELISAs using (a) anti-IgG1, (b) anti-IgG2a, (c) anti-IgG2b and (d) IgG3 as secondary antibodies.

Similar results were obtained with the recombinant protein i.e. only a significant IgG1 response was seen in mice injected with the recombinant RH HSP70 (Fig. 2a). The response to the recombinant protein was as high as that seen with the native protein confirming that the lack of proper conformation does not affect the immunogenicity of the recombinant. No significant IgG2a, IgG2b or IgG3 response was seen (Figs. 2b, 2c and 2d). Therefore, the response to the recombinant HSP70 was very similar to the response to the native protein. However, two facts arising from this study mitigate against the use of *T. gondii* RH HSP70 as a vaccine candidate. First, production of IgG1 indicates stimulation of interleukin 4 production and suppression of interferon-γ (indicative of a Th-2 response). This is contrary to current evidence, which suggests that interferon-γ is very important in the control of *T. gondii* infection (Alexander & Hunter 1998). Therefore, it seems that, although HSP70 may be important in the parasite response to the attack mounted by the host's immune system (Lyons and Johnson 1995), it does not seem to be one of the antigens provoking a protective immune response and so is of limited use as a vaccine candidate. Second, the overall response is fairly weak, indicating that the *T. gondii* RH strain HSP70 is not a very immunogenic protein. This is supported by the lack of response to HSP70 seen in serum from mice injected with a crude extract of *T. gondii* RH. This is surprising given the imuunogenicity observed in other parasite HSP70s such as those from *S. mansoni* and *S. japonicum* (Hedstrom et al. 1988). However, another study has found that anti-HSP70 antibodies reach only a modest peak at 1-2 weeks following infection of mice with an low virulent strain of *T. gondii* and then decline to be nearly undetectable after six weeks (Yui et al. 1998).

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REFERENCES

Alexander, J. & Hunter, C. A. 1998. Immunoregulation during Toxoplasmosis. *Chem. Immunol. 70:* 81-102.

Bianco, A. E., Favalora, J. M., Burkot, T. R. et al. 1986. A repetitive antigen of *Plasmodium falciparum* that is homologous to heat shock protein 70 of *Drosophila melanogaster. Proc. Natl. Aced. Sci. USA 83:* 8713-8717.

Hedstrom, R., Culpopper, J., Schinskl, V., Agabian, N. & Newport, G. 1988. Schistosome heat shock proteins are immunologically distinct host-like

antigens. *Mol. Biochem. Parasitol. 29:* 275-282.

Kaufmann, S. 1990. Heat shock proteins and the immune response. *Immunol. Today 11:* 129-136.

Kumar, N., Zhao, Y., Graves, P., Folgar, J. P., Maloy, L. & Zheng, H. 1990. Human immune response directed against *Plasmodium falciparum* heat shock-related proteins. *Infect. Immun. 58:* 1408-1414.

Laelmoli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:* 68-685.

Lyons, R. E. & Johnson, A. M. 1995. Heat shock proteins of *Toxoplasma gondii. Parasite Immunol. 17:* 353-359.

Lyons, R. E. & Johnson, A. M. 1998. Gene sequence and transcription differences in 70 kDa heat shock protein correlate with murine virulence of *Toxoplasma gondii. Int. J. Parasitol. 28:* 1041-1051.

Nandan, D. 1994. A rapid single-step purification method for immunogenic members of the hsp70 family: validation and application. *J. Immunol. Methods 176:* 255-263.

Sharma, Y. D. 1992. Structure and possible function of heat-shock proteins in Falciparum malaria. *Comp. Biochem. Physiol. 102:* 437-444.

Smith, N. C., Favila-Castillo,L., Monroy-Ostria, A., Hirunpetcharat, C. & Good, M. F. 1997. The spleen, IgG subsets and immunity to *Plasmodium berghei* in rats. *Immunol. Cell Biol. 75:* 318-327.

Towbin, H., Staehelin, T. & Gordon, J. 1978. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Aced. Sci. USA 76:* 4350-4354.

Wallace, G. R., Ball, A. E., MacFarlane, J., El Safi, S. H., Miles, M. A. & Kelly, J. M. 1992. Mapping of a visceral leishmaniasis-specific immunodominant B-cell epitope of *Leishmania donovani* HSP70. *Inf. Immun. 60:* 2688-2693.

Yui, K., Yamashita, K., Ueda, M., Udono,H. & Yano, A. 1998. Humoral immune responses against members of the HSP70 family in *Toxoplasma gondii. Parasitol. Int. 47 (Suppl.):* 272.