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HSP60, HSP70 and HSP90 from *Trichinella spiralis* as targets of humoral immune response in rats

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Abstract This study identifies three heat shock proteins (HSPs) using purified preparations from *Trichinella spiralis* larvae. The proteins: HSP60, HSP70 and HSP90 were found to be targets of the humoral immune response in rats. Three approaches were adopted to obtain *T. spiralis* HSP-enriched material and/or to purify HSPs to homogeneity. The former product was prepared using affinity chromatography on gelatin–sepharose and elution with ATP. Pure 90 kDa-protein was isolated from parasite extract by sequential DEAE (A50) column chromatograpy and preparative electrophoresis. Immunoblot analysis using monoclonal antibodies to HSP60, HSP70 and HSP90 detected the HSP60 and HSP70 in the affinity-purified product and HSP90 in the product obtained by sequential anionic chromatography and preparative electrophoresis. Finally, the reactivy of preimmune, *T. spiralis* immune and irrelevant immune rat sera on immunoblots were also examined. Only sera taken from infected rats at time-points after day 7 following the first infection exhibited activity against 60, 70 and 90 kDa proteins on blots. The fact that the serum antibodies were anti-HSP was established by immunoadsorption of HSPs to microtiter plates coated with anti-HSP60, anti-HSP70, or anti-HSP90 and using rat sera, positive on blots, to also give positive scores by continued enzyme-linked immunosorbent assay.

Keywords HSP · immunity · *Trichinella spiralis*

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Introduction

Over the past few decades, enormous advances have been made in our understanding of the roles and functions of heat shock proteins (HSPs). Increased understanding came to be realised by successive studies, each carried out to substantiate the value of some previous discovery in practice. Thus, the identification of HSPs as homeostasis perservers in defined stressful conditions (Lindquist 1986; Burel et al. 1992) stimulated studies indicating increased HSP expression in cells exposed to an 'endless' list of stimuli (Becker and Craig 1994). Previous studies have also suggested that HSPs per se, are indispensable for maintaining normal cell function (Schlesinger 1990) and directing the host's immune response (Gaston et al. 1989; Jacquier-Sarlin et al. 1994).

Three interesting associations have emerged from the above work. First, enhancement of HSP production is a prominent feature in all stressed cells. Second, HSPs are regulatory molecules involved in preventing loss of normal cell function and/or untimely cell death. Third, HSPs, despite their highly conserved nature, may be very immunogenic (Del Giudice 1994). These vital functions have led to the use of HSPs as: markers of injurious stress in disease (Macario 1995; Martinez et al. 1999a; Merino et al. 1998) and in drug efficacy studies (Tosi et al. 1997) and ecotoxicological studies (Eckwert et al. 1997); and as constituents of vaccines (Newport 1991). In a recent study on HSP60, HSP70 and HSP90 expression in organs of rats with a secondary *Trichinella spiralis* infection (Martinez et al. 1999b), we have established that such rats, contrary to primary-infected rats (Martinez et al. 1999a), exhibit no increased expression of test HSP. The mechanism of 'HSP non-responsiveness' in reinfected rats appears to relate to acquired immunity that seems to prevent occurrence of body-dwelling parasite stages. This discovery prompted us to examine whether *T. spiralis* HSP60, HSP70 and HSP90 are targets of the humoral immune response in the rat infection model.

The present study addresses this question using *T. spiralis* HSP-enriched material, or HSP purified to homogeneity; and post-infection and control sera. Notably, we exclude cross-reactivity to coincidentally similar-weight non-HSPs as the operative serum response and show that the serum responses displayed are infection-induced. Finally, we emphasize results obtained in a previous study, which demonstrated the HSP-nature of the three proteins purified or semi-purified in the present study using the same monoclonal antibodies for identification (Martinez et al. 1999c).

Materials and methods

Antibodies (Abs)

The monoclonal antibodies (MAbs) anti-HSP60 (clone LK-2), anti-HSP70 (clone BRM-22) and anti-HSP90 (clone AC 16) were from Sigma. Peroxidase-conjugated goat anti-mouse and goat anti-rat sera were obtained from Sigma and Nordic, respectively.

Collection and processing of *T. spiralis* larvae

GM1-strain *T. spiralis* muscle larvae (L_1) were obtained from NMRI mice according to reported procedures (Brand et al. 1952). Following washing of the larvae and resuspension in appropriate buffers (cf. HSP purification protocols), soluble whole L_1 extracts were prepared by procedures carried out at 4°C. These included homogenization of L_1 by repeated sonication, as well as by centrifugation at 100 000 *g* for 1 h. The resulting supernatant was collected, the protein concentration measured by Bradford procedure (Bio-Rad) before being stored in liquid nitrogen until needed.

Affinity chromatography (AffChr)

The procedure utilised in this study has been reported in detail (Nandan et al. 1994). Briefly, whole L_1 extract in buffer A (20 mM Tris, 0.15 M NaCl, pH 7.5) was applied to a gelatin–sepharose column (Pharmacia) equilibrated with buffer A and incubated for 2 h with gentle rotating. The column was then washed two times with buffer B (20 mM Tris, 0.5 M NaCl, pH 7.5) and once with buffer C (5 mM Hepes, pH 7.5), each washing step lasting 5 min. The adsorbed proteins were subsequently eluted using storage of the column in buffer D (3 mM ATP, 1 mM $MgCl_2$, 5 mM Hepes; pH 7.5) at room temperature (RT) for up to 2 h. Eluted proteins were concentrated using ultrafiltration membranes (30 kDa; Ultrafree, Millipore).

DEAE (A50) column chromatography (DeChr)

Soluble whole L1 extract in buffer l (50 mM Pipes, 0.1 M KCl, pH 6.8) was passed over an anion exchange column consisting of (3 g) DEAE-Sephadex A50 equilibrated with the same buffer. The adsorbed proteins were eluted in a stepwise manner from the column by successive washings with 0.2 M, 0.3 M and 0.5 M KCl at a flow-rate of 2 ml min^{-1}. The three fractions so obtained were concentrated and desalted by ultrafiltration.

Preparative electrophoresis (PrepE)

PrepE was performed using the Bio-Rad electrofractionation system (model 491 Prep Cell) as described (Martinez et al. 1998). Gels were prepared according to Laemmli (Laemmli 1970) and cast in a glass tube. Running gels (10%) were 50 mm and the stacking gel (4.5%) had a height of 5 mm. A peristaltic pump maintaining a constant flow rate of 0.5 ml min^{-1} was used to collect 3 ml fractions. The eluted protein fractions (i.e. in water) were concentrated by freeze-drying.

Analyses of purified HSP preparations

Purity of the HSP preparations was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and/or Western blotting.

Electrophoretic analysis and Western blotting

Purified proteins from *T. spiralis* larvae were separated by SDS-PAGE as described (Laemli 1970). Gels consisted of a 10% acrylamide separating gel and a 4% acrylamide stacking gel. Electrophoresis was performed in the cold room, at a constant current of 40 mA. Following SDS-PAGE, electroblotting of the separated polypeptides was done according to a slightly modified procedure (Towbin et al. 1979), encompassing the use of PVDF (polyvinylidene fluoride) instead of nitrocellulose as protein-supportive membrane (Martinez et al. 2000). Appropriate dilutions of the Abs (1/5000 diluted anti-HSP70, 1/1000 diluted anti-HSP60, 1/10000 diluted anti-HSP90 and 1/50 diluted rat serum) were then added. Blots probed with anti-HSP60 or anti-HSP90 were incubated for 2 h at RT, whereas those probed with anti-HSP70 were incubated overnight at 4°C. Following an incubation step with 1:6000 diluted peroxidase-conjugated anti-mouse serum, immunoreactions were visualized by incubation with a substrate comprising 0.06% 3,3′ diaminobenzidine and concentrated H_2O_2 diluted to a 1:1000 final dilution.

Rat sera

Sera analysed for anti-*T.spiralis* HSP-Ab were obtained from uninfected controls, primary-infected Wistar rats at days 1, 7, 14, 20 and 27 post-infection (PI), as well as from reinfected rats at days 7 and 14 post-reinfection (PRI). Each test group comprised three animals. All rats were free from contaminating intestinal parasitic infections, as assessed by weekly coprological screening.

Specificity testing procedure

Adsorption experiments were carried out on 96-well microtiter (Costar) plates. These were coated with 1/3000 diluted anti-HSP60, or anti-HSP70, or anti-HSP90 Ab in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.6, by storage overnight at 4°C. Following washing (3×) with PB-T and blocking of remaining active sites, adsorption of each test HSP species was obtained as follows. The HSP60 and HSP70-containing HSP preparation was added to the HSP60, as well as to the HSP70-capturing plate. The HSP90-containing HSP preparation was added to the anti-HSP90 coated plate. All HSP preparations were used at dilutions 1/500 in PB-T and plates were incubated for 2 h at 37°C. Experiments carried out prior to the present study have indicated a 2-h-incubation period to consistently remove all HSPs from the preparation. The ELISA (enzyme-linked immunosorbent assay) was then continued in order to test adsorded HSPs for reactivity with immune rat sera; the plates were washed three times with PB-T, incubated with immune rat sera in PB-T for 1 h at 37°C. The wells were subsequently incubated for 1 h at 37°C with peroxidase-conjugated goat anti-rat IgG and enzyme activity was measured with peroxide-substrate solution.

Results

Figure 1 summarizes the SDS-PAGE assays carried out on the purified *T. spiralis* preparations. The Coomassie

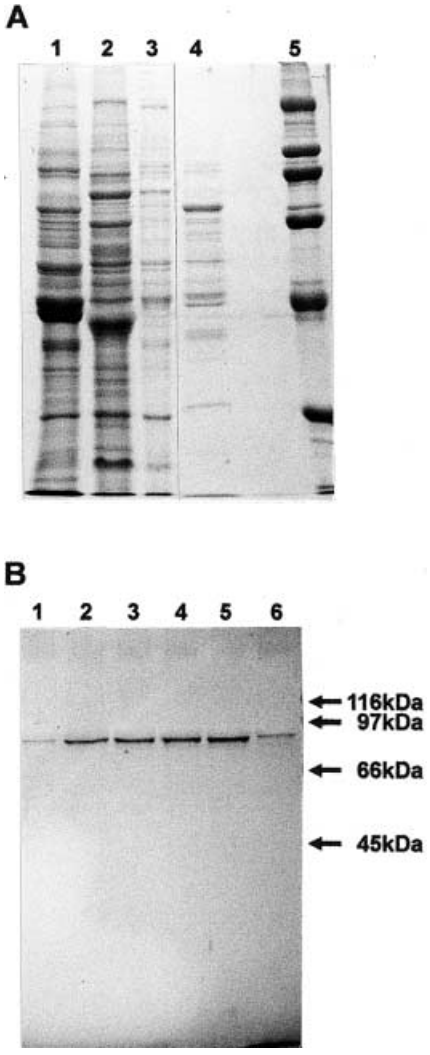

Fig. 1A–B SDS-PAGE analysis of *T. spiralis* HSP preparations. **A** Composite of data obtained in assays carried out using fractions obtained by DeChr (lanes 1–3) or gelatin AffChr (lane 4). Lane 1, eluted using 0.5 M KCl; lane2, eluted using 0.3 M KCl; lane 3, eluted using 0.2 M KCl; lane 4, eluted from the gelatin–sepharose column using ATP, and lane 5, molecular weight (205, 116, 97, 66, 45, 29 kDa) markers. **B** SDS-PAGE assays carried out using 6 consecutive fractions collected following further purification of the 0.3 M KCl-eluted fraction by PrepE. Relevant molecular-weight markers are indicated on the right with *arrows*

blue-stained polypeptide profiles of the fractions purified by DeChr (Fig. 1A, lane 1,2 and 3), or AffChr (Fig. 1A, lane 4), or silver-stained polypeptide profiles of six fractions purified by prepE (Fig. 1B) are illustrated. We shall focus on AffChr results because the method is supposed to isolate only HSP-like proteins and, therefore, a positive finding here is the most reliable indicator that a protein is an HSP.

As can be seen (Fig. 1A, lane 4), the AffChr 'HSP' preparation contained polypeptides of the following molecular masses: 111, 103, 78, 69, 68, 60, 57, 51, 47(2), 41, 37(2), 35 y 33 kDa. Relative amounts of polypeptides was in the order 78 kDa > > 47 kDa > 57 kDa≥37 kDa≥32 kDa. Immunoblot data of the preparation (i.e. also from more concentrated samples) (not shown) confirmed the presence of HSP60 and HSP70, but absence of HSP90. We thus conclude that gelatin–sepharose binds both the HSP60 and 70 species.

HSP90, as confirmed by Western blotting (not shown), was present in fractions eluted with 0.3 M and 0.2 M KCl from the DEAE-A50 column (Fig. 1, lanes 2 and 3, respectively). The fraction eluted using 0.3 M KCl being the most HSP90-enriched (cf. Fig. 1A, lane 2). Using the latter fraction for further purification by preparative electrophoresis revealed the elution of at least six fractions with anti-HSP90 activity, providing evidence that an almost 100% pure HSP90 preparation was obtained using anion exchange and subsequent preparative electrophoresis (Fig. 1B).

Figure 2 summarizes a representative SDS-PAGE/Western blotting assay from our gelatin–sepharose-purified fraction probed with sera from three rats in each of the subgroups indicated. As can be seen, rat preimmune sera already react against the 37 kDa and 47 kDa polypeptides. Note however, that increased reactivity to these two polypeptides occurred during the experiment, indicating *T. spiralis* infection has a boosting effect here. It was striking that (almost) all rats, starting from day 7 PI onwards, had additional strong serum responses to 110 kDa, 70 kDa and 60 kDa polypeptides in the preparation. Note also that the same PI and PRI sera (but not the preimmune sera) reacted with the 90 kDa protein purified by the combined AnChr-PrepE approach (Fig. 3).

That the above activity observed in PRI rat sera against purified preparations included activity due to (also) contained anti-HSP60, HSP70 and HSP90 serum Ab was verified by the following set of experiments. The relevant proteins contained in our two 'HSP-enriched' preparations (cf. Fig. 1A, lane 4 and Fig. 1B) were retained using antibody-coated ELISA plates which were thereafter probed with all test rat sera and developed.

All sera (diluted 1:100) from three rats in each of the six positive p.i. groups (see Figs. 2 and 3) gave positive reactions (optical density values at 405 nM (OD_{405})≥ 0.750) with the plate-adsorbed HSP60, HSP70 and HSP90. No positive ELISA scores ($OD_{405} \leq 0.245$) were noted with preimmune serum, or day 1 PI rat sera, or an irrelevant immune rat serum (containing anti-bacterial Ab).

Discussion

Firstly, we want to emphasize that we are confident about the identification of the three proteins immunologicaly studied (HSP90, HSP70 and HSP60) because there are three signs indicating that we are working with the HSPs in question: (1) the proteins are induced in response to several stressors (Martinez et al. 1999c); (2) they are recognized by the same specific MAbs used in the above-mentioned work; and (3) immunoreactivity

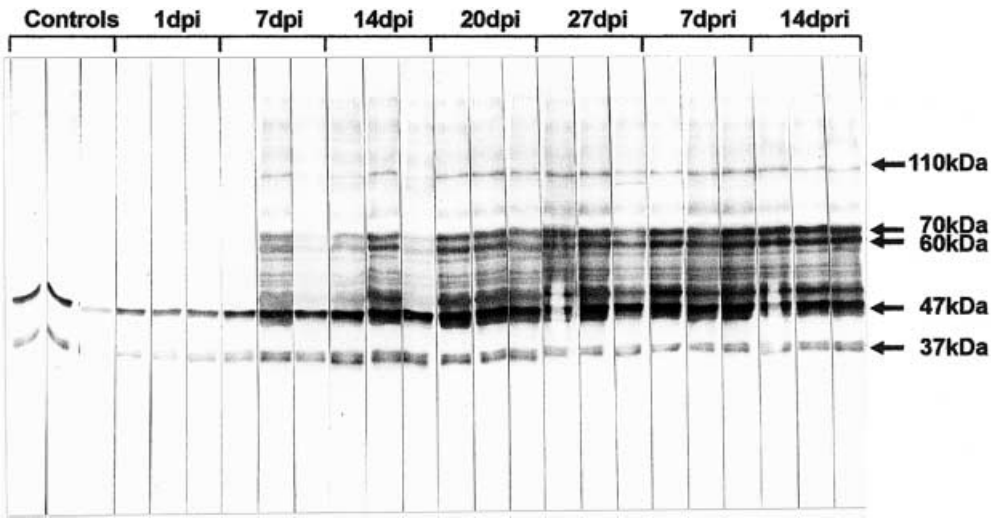

Fig. 2 Western blot of affinity-purified *T. spiralis* HSPs reacted with rat preimmune (control) and immune serums. The reactivity of sera from three different rats obtained at the indicated day postinfection (PI) or post-reinfection (PRI) is presented. Molecular-weight markers are indicated on the right with *arrows*

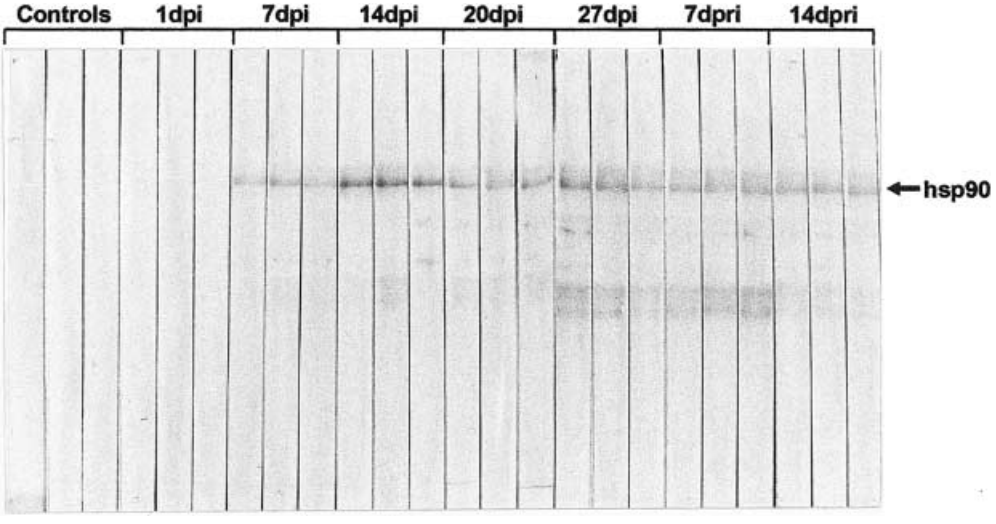

Fig. 3 Western blot of *T. spiralis* HSP90 purified by combined DeChr + PrepE approach (see text) and reacted with rat preimmune (control) and immune serums. The reactivity of sera from three different rats obtained at the indicated day post-infection (PI) or post-reinfection (PRI) is presented. Molecular-weight markers are indicated on the right with *arrows*

showed by MAbs coincides with the molecular weight of those proteins.

The objective of this work was twofold. First, we used AffChr and two other methods to determine if these choices could produce *T. spiralis* HSP-enriched preparations (containing particularly HSP60, HSP70 and HSP90). Second, in achieving this goal, we wanted to ascertain whether PI rat sera reacted with any of these HSPs in order to establish their immunogenicity during infection. These three HSP purification methods were chosen for the following reasons: (1) gelatin AffChr is a method shown to allow for the purification of members of the HSP70 family from all tissues studied (Nandan et al. 1994) and should, therefore, also be included here; (2) the anion exchange method, by enabling fractionated elution of bound proteins, afforded for the preparation of fractions enriched for HSP90; (3) PrepE (Martinez et al. 1998) of the above HSP90-enriched preparation allowed us to obtain an almost 100% pure HSP90 preparation.

According to the Nandan et al. (1994) study, comparing several gelatin-purified preparations for constituent proteins, the latter are HSPs of either the HSP78 or HSP70 class, albeit that (inducible) HSP47 was sometimes also noted. These molecular weight proteins were also detected in our purified preparation, as were several other molecular weight proteins, including a 60 kDa protein. Immunoblot analyses using anti-HSP60, anti-HSP70 and anti-HSP90 Ab confirmed the presence of HSP60 and HSP70, but not HSP90, in this preparation. It should be noted that detection of HSP60 here indicates *T. spiralis* HSP60 can be obtained by gelatin AffChr. Two characteristics of the proteins present in the above preparation suggest their possible function as chaperone molecules: (1) they bind denatured proteins, such as gelatin (i.e. denatured collagen); and (2) proteins are eluted from the columns using ATP. In this preparation we could conclusively demonstrate that two out of the 11 proteins present were also HSPs (see above). Based on reported data indicating HSP47 and HSP78 from many tissue sources bind to gelatin–sepharose (Nandan et al. 1994), it is likely that the 78 kDa and 47 kDa proteins found in our purified preparation are

also *T. spiralis* HSPs. The present study did not include immunological examination of this preparation with either anti-HSP47 or anti-HSP78 Ab; therefore, we cannot directly address this question. The same applies to the other molecular weight proteins present in the preparation. Also no other approaches for verifying that proteins present in our gelatin–sepharose derived preparation were indeed HSPs were available. A clear limitation of our study is that the method for collecting *T. spiralis* L_1, by itself, causes enhanced HSP expression. This clearly impedes the, for example, willful application of a stimulus, to pinpoint consequentially produced *T. spiralis* HSPs.

After obtaining data that strongly suggested we had produced two HSP-containing preparations, we designed an experiment to study antigenicity of *T. spiralis* HSPs in rats with this helminth infection. Although the exact nature of several proteins (HSPs, or not) in our Affchr-purified preparation remained uncertain, several reactions of our test sera to the proteins contained therein deserve to be mentioned. First, we observed that all uninfected rat sera already reacted with a 37 kDa and 47 kDa protein and that boosted reactions to these proteins occurred PI and PRI with *T. spiralis*. These serum responses were also observed in day 1 PI sera and three irrelevant rat immune sera (data not shown). Serum reactivity against these two proteins is, thus, a very general feature of normal rat sera and is apparently due to an induced response to a self (rat) antigen. In view of the cross-reactivity with similar molecular-weight (and gelatin-binding) proteins (and therefore, presumed HSPs), it is tempting to speculate that normal rats manifest anti-(self) HSP37 and HSP47 responses. The biological consequences, if any, of such spontaneously occurring responses are unclear.

The second and most striking observation was that, from day 7 PI onwards, all infected, but also all reinfected, rat sera reacted strongly with 60 and 70 kDa proteins in the affinity-purified preparation and less strongly with a 90 kDa protein in the prepE-purified preparation. That these Abs were actually anti-*T. spiralis* HSP60, HSP70 or HSP90, was verified by our continued ELISA test using PI and PRI rat sera to specifically react with either *T. spiralis* HSP90, or HSP70, or HSP60 adsorbed to HSP-specific Ab. This finding indicates *T. spiralis* infections cause the induction of, at least, anti-HSP60, anti-HSP70 and anti-HSP90 serum Ab and that, therefore, these HSPs might be important targets of the humoral immune response. Further studies are required to define the biological consequences of such responses.

In conclusion, the present study describes the purification or semipurification of *T. spiralis* HSPs and identifies HSP60, HSP70 and HSP90 as probable targets of the immune response in the host with this helminth infection. A larger study will be necessary to evaluate further the effects of such immune responses on host–parasite relations. From a biological point of view, we propose that the various HSPs defined here may contribute to different aspects of the host response to *T. spiralis* infection. Our studies (Martinez et al. 1999a; Martinez et al. 1999b) indicate that such HSP-dependent regulatory mechanisms might be operative in our *T. spiralis* rat infection model.

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