Shock response induced in rat brain and spleen during primary infection with *Trichinella spiralis* larvae

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SUMMARY

An infection approach was adopted for examining consequential heat shock (HS) or stress response in brain and spleen tissues from Wistar rats. Stress in this system was due to interactions with the infecting helminth, *Trichinella spiralis*, or its body-dwelling larval stages, or products thereof. It was argued that in the infection model used, elements effecting stress in the brain would differ from those in the spleen. HS responses were measured by quantitation of 4 levels of HS proteins (HSP25, HSP60, HSP70 and HSP90) with time, in infected and uninfected rat tissues using an assay depending on immunoblotting specifically to detect the separate HSPs and image analysis to measure HSP content. In brain and spleen tissue from uninfected rats, a continuous expression of the above HSPs was observed at levels which hardly varied throughout the experiment. In contrast, HSP25, HSP60 and HSP70 levels in infected rat tissues varied and apparently depended on 'infection cycle'-related events. Thus, an enhanced expression of HSP25 and HSP60 and of these plus HSP70 was observed at certain, yet different, time-points during infection in rat spleen and rat brain, respectively. Interestingly, HSP90 expression in spleen tissue from infected rats versus controls, was significantly reduced throughout the experiment suggesting some important (as yet undefined) role for HSP90 in the infection cycle. These studies seem to have provided evidence for the occurrence of soluble factors causing altered HSP expression at both sides of the blood-brain barrier in rats with a primary *T. spiralis* infection.

Key words: HSP, nematode, *Trichinella spiralis*.

INTRODUCTION

All organisms exposed to a stressful condition exhibit the so-called heat shock (HS) response (Lindquist, 1986; Polla, Perin & Pizurki, 1993). This response characteristically involves the activation of HS genes and subsequent translation of HS RNA to produce heat shock proteins (HSPs). It has been reported that HSPs act as molecular chaperons for protecting cells against further injury induced by the stressful agent, or condition (Schlesinger, 1990). Other important functions include a role in protein synthesis (Ellis & Hemmingsen, 1989) and in protein targeting for lysosomal degradation (Terlecky, 1994), or antigen processing (DeNagel & Pierce, 1992) and to induce microfilament stabilization (Liang & MacRae, 1997) and protective immunity (Jacquier-Sarlin *et al.* 1994).

Enhanced HSP production by exposure to thermal stress, i.e. the classical HS response, is now recognized as one form of a more general stress response. To date, many more stressors are known to induce classical HSPs along with other so-called stress-specific proteins (Macario, 1995). They include, for example, certain drugs, reactive oxygen species, cytokines and heavy metals (Lindquist, 1986; Polla, Perin & Pizurki, 1993) as well as protozoans (Lindley, Chakraborty & Edlind, 1988; Himeno, Nagasawa & Hisaeda, 1993) and helminth parasites (Hedstrom *et al.* 1987; Rothstein *et al.* 1989).

Excepting the above authors who principally studied HSP function as immunogens, HSP production and other HSP function(s) in hosts with helminth infections have, to our knowledge, received little, if any, attention so far. In this respect, the *Trichinella spiralis*–rat model seems to be an appropriate candidate for study because here overt (and differential) injury to organs occurs as the host goes on to become immune (Bullick, Russell & Castro, 1984; Appleton & McGregor, 1987). It is therefore likely that HSP expression during a primary *T. spiralis* infection will reflect tissue damage, the parasite's evasive actions as well as the host's defensive responses.

Our previous work in this field has utilized the Western blotting technique and image analysis to

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measure altered expression of HSPs in the liver and muscle of rats with a primary infection (Martinez *et al*. 1999). However, liver, and especially muscle, are target organs for *T. spiralis* larvae and the finding of altered HSP production in these organs is therefore not unexpected. The availability of organs outside the normal route of migration by *T. spiralis* larvae, prompted us to use some of these organs to examine the HS response, if any, therein during infection with this parasite.

Two organs from rats were used. The first was the brain, which is a tissue only rarely visited by body-dwelling newborn larvae (NBL) of *T. spiralis* (Schope, 1949; Weatherly, 1983). In contrast, the other organ, the spleen, is more prone to have stray NBL and usually presents signs of organ distress in the host with trichinosis (Weatherly, 1983). In the present study, we measured HSP25, HSP60, HSP70 and HSP90 levels in brain and spleen tissue of both uninfected rats as well as in rats with a primary *T. spiralis* infection.

MATERIALS AND METHODS

Reagents

The supportive membrane PVDF (polyvinylidene fluoride) was purchased from Millipore (Inmobilon P). The non-pyrogenic, isotonic solution (NPIS) used in the study was from Pharmacia. Defatted milk powder was purchased from Nestle. PBS with 0·05 % Tween 20 (PB–T) was routinely used. The blocking buffer consisted of PB–T + 5 % (w/v) defatted milk.

Antibodies (*Abs*)

The monoclonal antibodies (mAbs) anti-HSP60 (clone LK-1) and anti-HSP70 (clone BRM-22) were from Sigma. The mAb anti-HSP90 (clone AC88) and the polyclonal antibody anti-hsp25 (immunogen: recombinant mouse hsp25) were from Stressgen. Peroxidase-conjugated goat anti-mouse serum was obtained from Sigma.

Parasite infection and collection of experimental tissues

Female Wistar rats, weighing approximately 200 g were used in this study. They were obtained from the University's breeding facility (Alcala de Henares University, Spain), were housed in well-ventilated isolation rooms with programmed illumination, and were provided food and water *ad libitum*. On day 0, all rats were inoculated orally with 1 ml of NPIS only (controls) or NPIS containing 6000 fresh L_1 (muscle) larvae of *T. spiralis* of the GM-1 strain. The (L_1) larvae used herein were obtained using described procedures (Brand *et al*. 1952) and extensively washed with NPIS before inoculation. When infected with 6000 larvae, our routine infection protocol for Wistar rats which basically follows the Bullick *et al*. (1984) infection protocol (7000 L1/rat), the rats, both clinically and behaviourly, showed no overt signs of infection dose-related distress (such as diarrhoea, uneasiness, etc); they thrived and behaved as did the uninfected controls.

At 1, 7, 14, 20 and 27 days post-infection (p.i.), 4 infected rats and 1 control rat were anesthesized and killed by cervical dislocation. Immediately after sacrifice the brain and spleen of each rat were removed, washed extensively with 0·9 % saline and stored in liquid nitrogen until processed.

Processing of rat tissues

All procedures used for obtaining rat specimens for analysis of HSP production were carried out at 4 °C. These included homogenization of the rat tissues (T25 Standard Euroturrax) and repeated sonication to disrupt all cells as well as centrifugation at 13 100 ***g*** for 20 min. The resulting supernatant was collected, was analysed for protein content by the Bradford procedure (Bio-Rad) and was stored in liquid nitrogen until needed.

Electrophoretic analysis and Western blotting

Proteins in rat specimens were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) procedure as described (Laemmli, 1970). Gels were composed of a 10 % acrylamide separating gel and a 4 % acrylamide stacking gel. Samples, each with an equal amount of protein (0·03 mg) were loaded in the lanes and 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 Towbin, Staehelin & Gordon (1979) procedure encompassing the use of PVDF (Inmobilon P) instead of nitrocellulose as the protein-supportive membrane.

The PVDF membranes were then (and also between all the below incubations) washed with PB–T for 10 min. Blots were blocked using blocking buffer and incubation for 1 h. Appropriate dilutions of the Abs (1:1500 diluted anti-HSP25 and anti-HSP60; 1:5000 diluted anti-HSP70 and 1:1500 diluted anti-HSP90) were then added and blots were incubated for 1 h at room temperature. 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.

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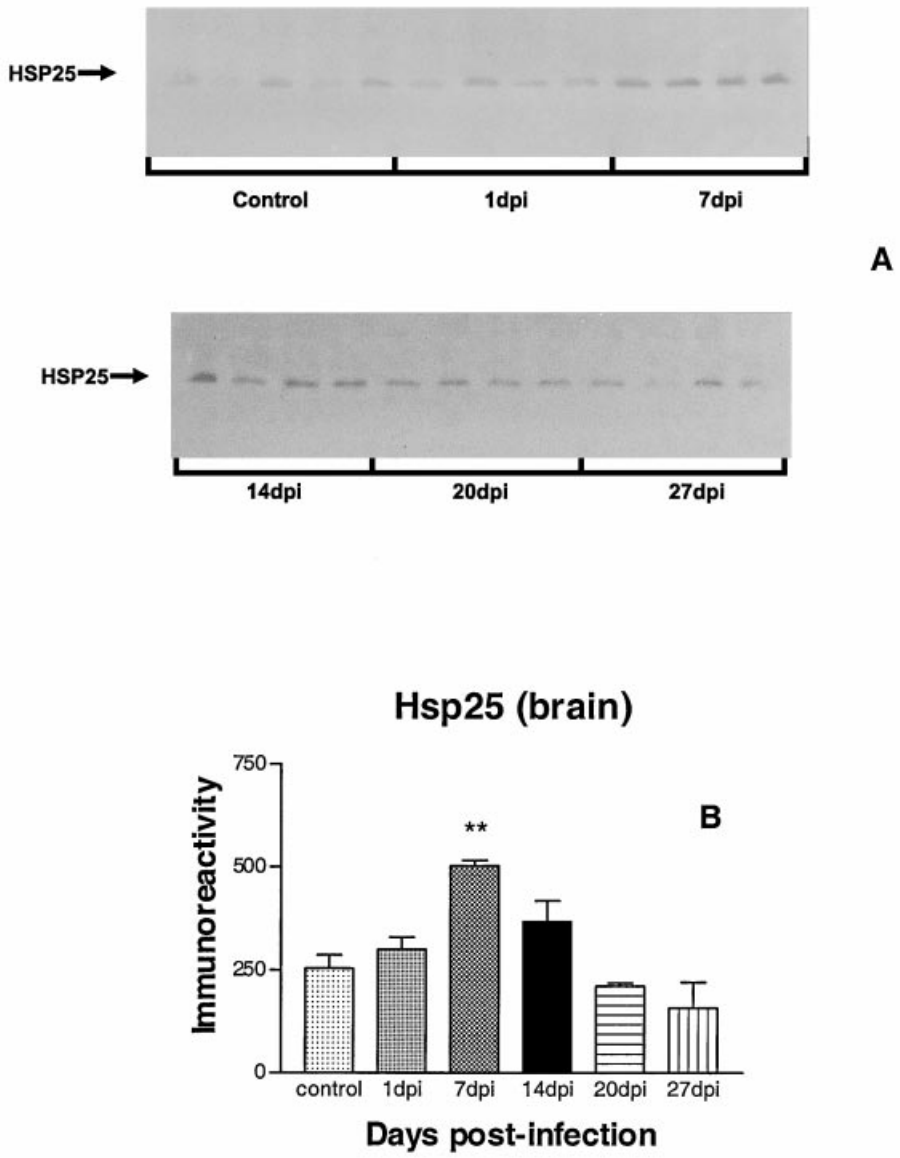

Fig. 1. Analysis of HSP25 immunoreactivity in rat brain. (A) Western blot analysis using polyclonal antibody anti-HSP25. (B) Densitometric analysis of HSP25 immunoreactivity (**$P < 0{\cdot}01$).

Statistical analysis

After immunoblotting, the immunoreactivity of the various samples was quantitated using an image analyser (Program MIP 1.6. Microm Spain). The differences in immunoreactivity observed between samples from control rats and those from infected rats were analysed by the Dunnet test.

Linearity of Western blot test results

Prior to this study experiments were carried out to demonstrate linearity of immunoblot results under the conditions to be used herein. Recovery of each HSP by its HSP-specific Ab to be employed in the Western blot was assessed by assaying rat brain and spleen specimens with varying amounts of proteins. Evaluation of test results on these samples by regression analysis, in all cases, revealed a very high correlation ($r > 0{\cdot}95$) between observed immunoreactivity and amount of protein applied in the test.

RESULTS

HSP production in rat brain

Brain specimens obtained on each of days 1, 7, 14, 20 and 27 p.i. from 4 *T. spiralis*-infected rats and 1 non-infected control were all assayed at once for either HSP25, HSP60, HSP70, or HSP90 production. As regards HSP25 and HSP60 production, no significant differences were seen in these stress parameters between infected rats and controls before second sample collections. At the second time-point (day 7 p.i.), infected rat brain had significantly more HSP25 than controls, the level having returned to

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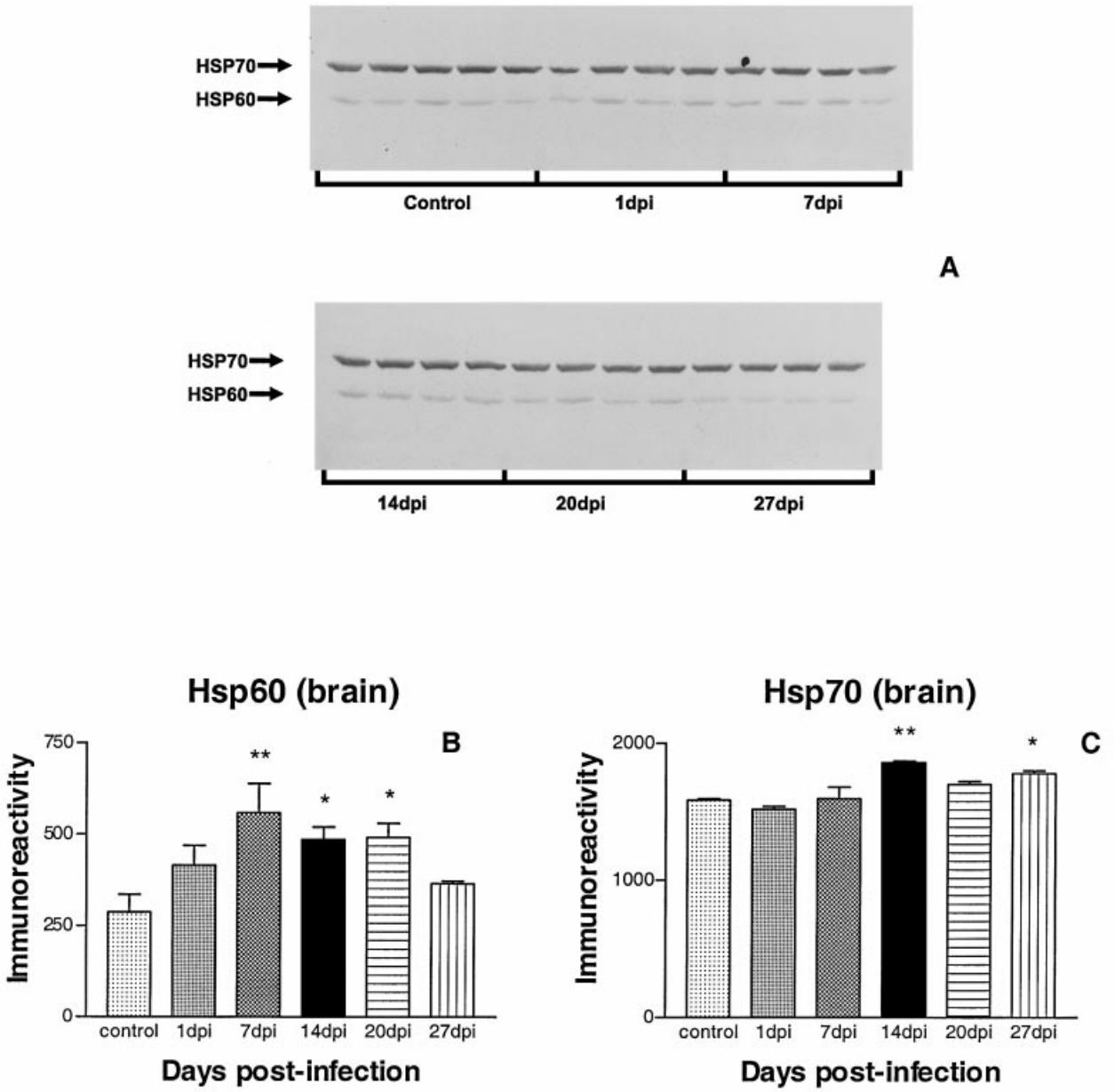

Fig. 2. Analysis of HSP60 and HSP70 immunoreactivity in rat brain. (A) Western blot analysis using monoclonal antibodies anti-HSP60 and anti-HSP70. (B) Densitometric analysis of HSP60 immunoreactivity (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$). (C) Densitometric analysis of HSP70 immunoreactivity (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$).

normality already at the last collection date (Fig. 1A, B). In contrast, in infected rats significant enhancement of HSP60 production was found at day 7 p.i. which persisted for $\geqslant 2$ weeks and thereafter returned to normality (Fig. 2A, B). No significant differences were found in HSP70 production between infected and control rats at the first and second sample collections. At the third time-point (day 14 p.i.), infected rat brain specimens had significantly more HSP70 than controls, this significant trend and/or difference remaining during the experiment (Fig. 2A, C). In contrast, no significant differences were found in HSP90 expression between infected and control animals at any time-point during the experiment.

HSP production in rat spleen

Spleen specimens from the same rats were assayed at once for either HSP25, HSP60, HSP70, or HSP90. In inoculated rats, there was a significant increase in HSP25 production in the spleen at day 1 p.i. when compared with that of the control rat, but production thereafter was similar to that of controls (Fig. 3A, B). No significant difference in HSP60 production was found between infected and control rats at the first, second and third sample collections. At time-point day 20 p.i., however, the spleen specimens from infected versus control rats had significantly more HSP60 than did that of the control rat, but at the collection dates thereafter HSP60

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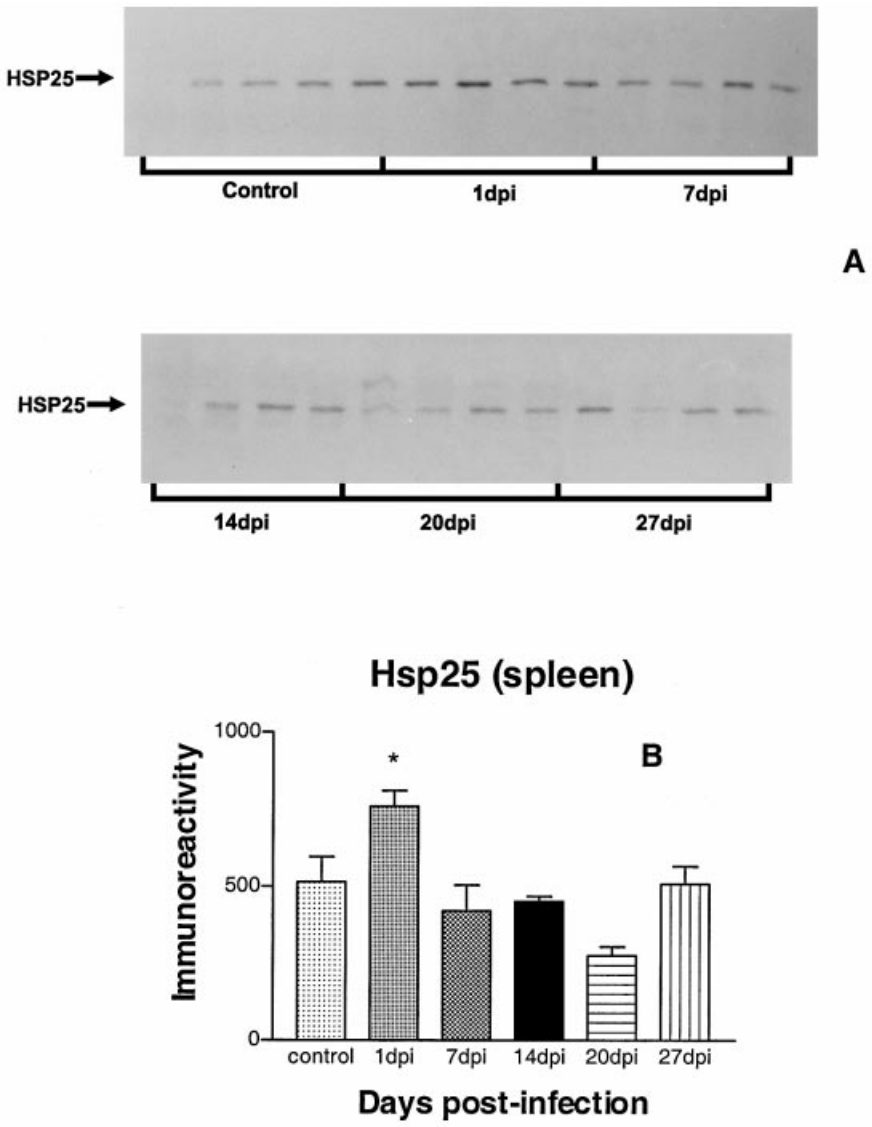

Fig. 3. Analysis of HSP25 immunoreactivity in rat spleen. (A) Western blot analysis using polyclonal antibody anti-HSP25. (B) Densitometric analysis of HSP25 immunoreactivity (*$P < 0{\cdot}05$).

production in the 2 groups, once again, did not differ (Fig. 4A, B). In contrast, there was no significant difference between infected and control rats in HSP70 production at any time during the experiment (Fig. 4A, C). On the other hand, mean HSP90 expression results, although rather variable, at all time-points were significantly lower in infected rats than in controls (Fig. 5A, B). This suggests that relative translation of HSP90 RNA is subject to down regulation in *T. spiralis*-infected rats.

DISCUSSION

The present study shows that *T. spiralis* infection significantly enhanced HSP25, HSP60 and HSP70 production in brain tissue, but not the HSP90 production. To our knowledge, this is the first study showing that in rat trichinellosis, the brain, in absence of dwelling parasites, is being programmed to increase the production of certain HSPs. Altered expression of HSPs in brain tissue due to exposure to a variety of stresses has been described earlier (Brown, 1990; Plumier *et al.* 1997), strongly suggesting a role as regulatory 'events' in either protection against further injury by the stressor, or in maintenance of the induced protection. The situation with regard to the role(s) and function(s) of HSP expression in the brain of *T. spiralis*-infected organisms, because of lack of past experience, is less clear. Obviously, all or some of the HSPs detected in increased amounts during this study in brain tissue of inoculated rats, may function (to mention only the extremes) to either facilitate muscle larvae encystation, or to induce resistance to reinfection. Another study should address both possibilities.

The increased production of HSP25 at day 7 p.i. coincides with release of NBL in the circulation (Harley & Gallicchico, 1971; Despommier, 1983). Although the majority of NBL head for the striated muscle of the host to transform into encysted muscle

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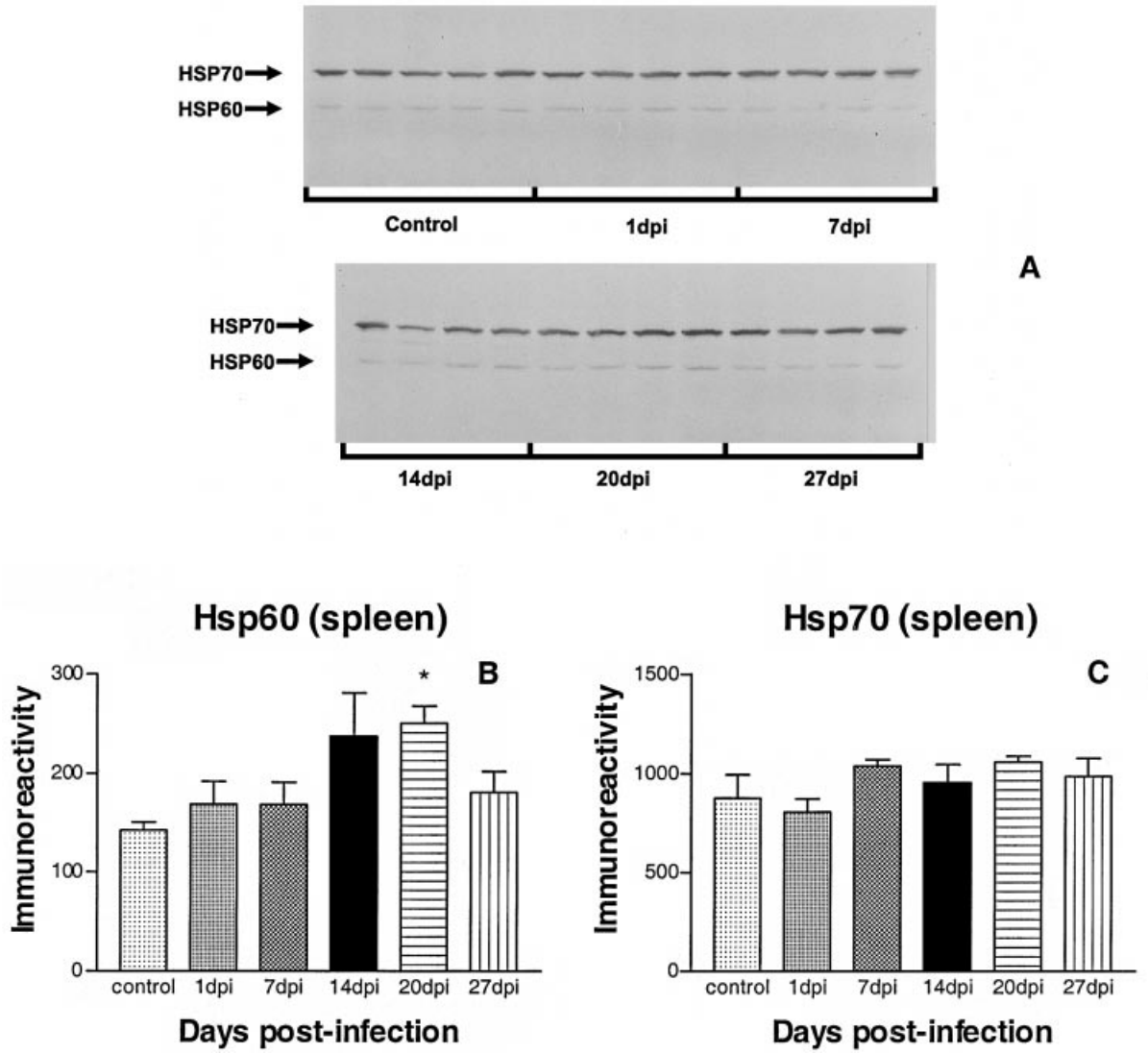

Fig. 4. Analysis of HSP60 and HSP70 immunoreactivity in rat spleen. (A) Western blot analysis using monoclonal antibodies anti-HSP60 and anti-HSP70. (B) Densitometric analysis of HSP60 immunoreactivity (*$P < 0{\cdot}05$). (C) Densitometric analysis of HSP70 immunoreactivity.

larvae, a few have also been detected in both the cerebral fluid as well as the brain tissue (Schope, 1949). Thus, encephalitis and encephalomeningitis are clinical (yet rare) features in human trichinellosis (Weatherly, 1983). Nevertheless, it has been presumed that brain injury is not due to direct NBL action but to toxic effects of NBL metabolites (Edwars & Hood, 1962). A possible and likely candidate material capable of causing such adverse reactions *in vivo*, is the excretory–secretory (E–S) material shown to be present in culture media harbouring NBL (Gamble *et al*. 1988).

Significantly enhanced HSP60 production, contrary to enhanced HSP25 production which was observed at collection day 7 p.i. only, was maintained for 2 more weeks. A similar prolonged enhancement of HSP70 production was observed in the brain of infected rats, albeit that enhanced production of the protein was first observed at time-point day 14 p.i. The different kinetics of the above HSPs in the present study (still) remains to be explained. Several possibilities exist including presence of different stresses at different time-points of the parasite's life-cycle and/or the 'programmed' need (parasite, or host-mediated) of brain cells for different HSPs at different time-points during the infection. In this regard also the observation that host body temperature rises prior to NBL being released into the circulation between days 7 and 9 p.i. (Beaver, Jung & Cupp, 1986) should be considered a factor that may cause the observed enhanced expression of HSP by brain cells. Taken in conjunction with our present study, these results seem to suggest that enhanced expression of HSP (but also the persistently down-regulated expression of HSP90 observed herein) in infected rats, is regulated by molecules that should easily cross the blood–brain barrier and probably be contained in the NBL culture medium.

The enhanced production of HSP25 in rat spleen at day 1 p.i. is both surprising and interesting

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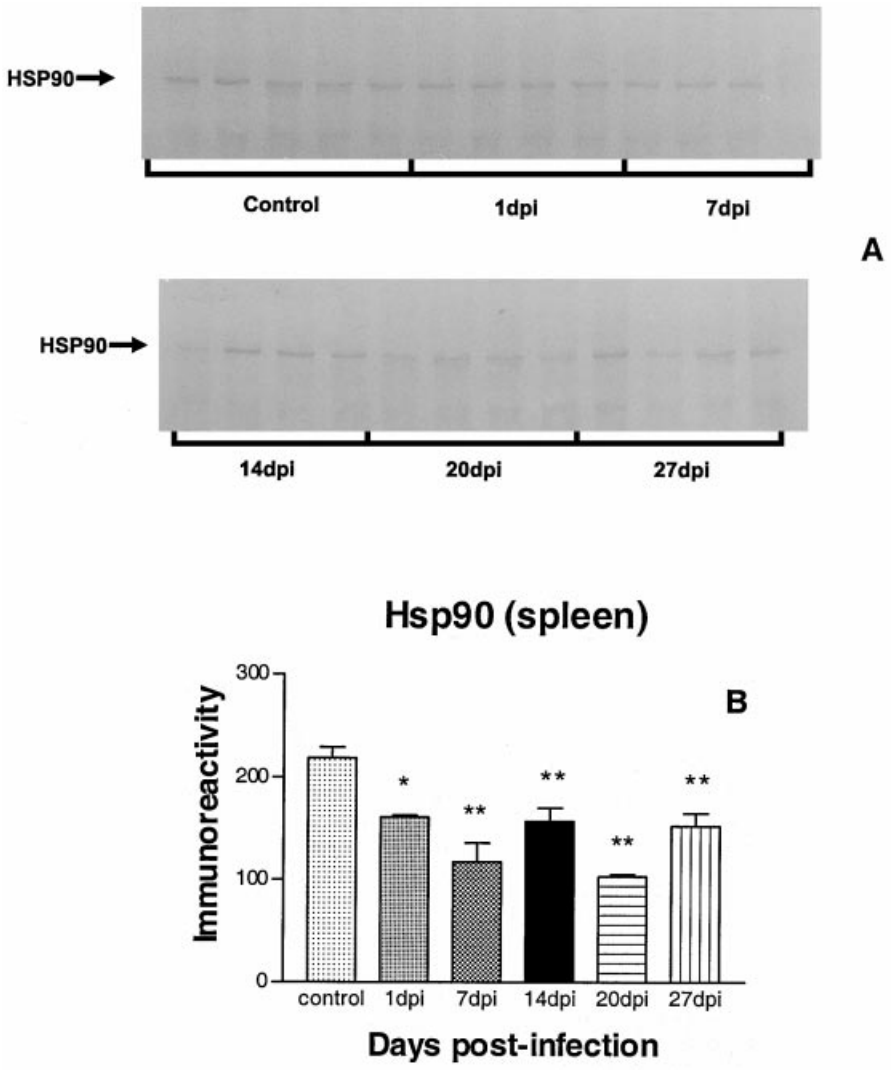

Fig. 5. Analysis of HSP90 immunoreactivity in rat spleen. (A) Western blot analysis using monoclonal antibody anti-HSP90. (B) Densitometric analysis of HSP90 immunoreactivity (*$P < 0{\cdot}05$, **$P < 0{\cdot}01$).

because 1 day after infection with *T. spiralis* this parasite has definitely not been in physical contact with spleen. One day after infection the parasite still resides in the intestines of the host as either (predominantly) L_1 infective larvae, or (a few) adult worms (Despommier, 1983). In all, these data strongly suggest that enhanced production of HSP25 in rat spleen 1 day after *T. spiralis* is definitely brought about by parasite-related factors that cause stress to the spleen in an indirect manner. In view of the very early onset of this HS response, it is tempting to believe that early larval E–S products have a role in the induction of the observed phenomenon. Injection of L1-larval E–S products, or day 1 post-infection serum into naive rats should confirm (or reject) this assumption (and also explain why this feature is not shared by all organs). Another mediator of the observed phenomenon might be the induction of fever in the host by the experimental inoculum. If this were to have been the case in the present study, then a difference in body temperature between non-infected rats and the infected rats should exist. However, we did not find any difference between *T. spiralis*-infected and non-infected control rats when body temperatures on days – 1, 0 (day of infection) and 1 p.i. were compared (data not shown). The implication here is that another mediator of the observed HSP response in spleen in infected rats on day 1 p.i. must exist.

Bouchama *et al.* (1991) and others have shown that exposure of intestinal cells to thermal stress results in the release of endotoxins. Given the knowledge that most classical HS responses are inducible by many stressors other than heat (Lindquist, 1986; Polla *et al.* 1993; Macario, 1995), it may reasonably be argued that endotoxin production is a prominent feature in the host with intestinal *T. spiralis*. Since endotoxins are known stressors it is then possible that the factor underlying the enhancement of HSP25 production in the spleen of rats 1 day after *T. spiralis* infection, is endotoxin originating from the host's intestine, or even from stressed L_1 larvae therein. Such hypothetical (yet not far-fetched) mechanism of HSP induction 1 day after infection

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may, in the light of evolution, be of considerable interest as the induced HSPs may help to prepare the spleen for coming events.

Although NBL are usually not detected in spleen tissue, this organ exhibits hyperplasia and hyperaemia in the host with trichinosis. Probably these clinical signs result from a general inflammatory process and the induced immune responses against *T. spiralis* antigens (Weatherly, 1983). For example, increased specific IgG occurs in the spleen in inoculated rats at day 14 p.i. (data not shown), which coincides with a substantial enhancement of HSP60 production towards a level significantly different from that of controls detected 1 time-point thereafter. HSP60 may have a role in the regulation (or modulation) of the inflammatory response in the spleen, an (as yet) hypothetical function recently reported to hold true for HSP25 (Moseley, 1997).

Taken together, our data strongly suggest that in the *T. spiralis*-infected rat, at both sides of the blood–brain barrier, cells programmed to make HSPs proteins are triggered to produce HSPs at levels 'required'. In the case of the brain, small parasite-derived molecules (and not the migrating parasite *per se*) likely play an important role in HSP expression. Insight into the parasite-related elements affecting the expression of which HSP(s) in which organ, and to what extent, may aid in determining which organs suffer most during infection, thus facilitating (i) the completion of proper (organ-targeted) therapies and/or (ii) the establishment of new diagnostic criteria. Such effects when attainable, may certainly show favour to individuals with trichinosis but maybe also to individuals with other parasitosis.

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