Prior immunity to *Trichinella spiralis* prevents (re)occurrence of an explicit stress response in intestines but not in mesenteric lymph nodes, heart and lungs from reinfected rats

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SUMMARY

We recently showed that, in our *Trichinella spiralis* rat model, first exposure, but not re-exposure to infective-stage larvae evoked heat shock responses in 4 test organs. Our work, however, failed to implicate either early complete clearance of challenge muscle larvae (ML), or rapid elimination of newborn larvae (NBL) in the phenomenon noted in reinfected rats. This study clarifies that issue using 2 established facts in *T. spiralis* biology and anti-*T. spiralis* immunology. That is, adult worms injure gut cells and immune destruction of NBL requires release of material also toxic to host cells. To approach the above problem we analysed relevant and irrelevant rat organs for increased heat shock protein (HSP) production at 1, 7, 14, 20 and 27 p.i. during first and second infections. Organs examined were intestines, mesenteric lymph nodes (MLN), heart and lungs. Using densitometric analyses of immunoblots, increased HSP expression was detected on day 7 in intestines from both primary and secondary-infected rats albeit that the change in the latter was just short of significant. Interestingly, MLN only exhibited increased HSP levels in the reinfected rat model with increased HSP levels persisting for 1 week. A lasting shock response was detected in reinfected rats; in contrast, first exposure resulted in shock responses being evident in lungs at either day 7 or day 14, only. These findings suggest that (i) in immune rats, a few challenge ML develop into adults, produce NBL which are trapped within MLN, and (ii) that anti-*T. spiralis* and/or anti-NBL immunity is associated with an, as yet, uncomprehended stress to host's heart tissues.

Key words: heat-shock protein, heart, lung, mesenteric nodes, *Trichinella spiralis*.

INTRODUCTION

Analyses of cellular and organ HSP production is an approach now widely applied in life sciences to studying various processes. Two findings furthering our understanding of HSPs established good grounding for this approach. The first indicated enhancement of HSP production to be a feature of all stressed cells (Lindquist, 1986; Welch, 1992; Macario, 1995). The second identified HSPs as molecules central to the maintenance of cellular homeostasis, and hence, of cell function (Schlesinger, 1990; Leppä & Sistonen, 1997). A rapidly expanding number of studies, each applying HSP level determinations for a different reason, ensued. For example, HSP expression has been used in ecological (Koehler & Eckwert, 1997; Merino *et al*. 1998), pathological (Yellon & Marber, 1994), chemotherapeutical (Tosi *et al*. 1997), immunological (Lamb & Young, 1990; Mollenhauer & Schulmeister, 1992) and parasitological (Maresca & Carratú, 1992; Maresca & Kobayashi, 1994) studies.

We have recently shown that rats with a primary *Trichinella spiralis* infection manifest increased HSP expression in internal organs situated both along and outside the normal migration route by *T. spiralis* NBL (Martínez *et al*. 1999*a*, *b*). In contrast, no altered HSP expression was noted in rats with a secondary infection with this nematode (Martínez *et al*. 1999*c*).

During the above work, we established that immunized rats rapidly expel 90–95% of challenge ML from their guts. These studies have also indicated ML burden in reinfected rats to equal that of primary-infected rats, suggesting a 0% establishment of migratory NBL (if any) in immune rats. It has been reported that *T. spiralis* immune rats manifest both anti-L_1 and anti-NBL immunity (Wang & Bell, 1987). Therefore, 2 processes might account for the aforementioned phenomenon of unaltered ML burden and HSP expression in our

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Table 1. Muscle larvae burden in test and challenge control rats

Group*	Treatment: Sensitization (day 0)	Treatment: Challenge (day 45)†	Number of muscle larvae established (×1000)‡
1 ($n = 6$)	6000 L_1§	6000 L_1	231·5 ± 55·7¶
2 ($n = 7$)	6000 L_1	—	202·5 ± 83·6
3 ($n = 2$)	—	6000 L_1	238 ± 43·5

* Group 1 included all reinfected rats killed at days 20 + 27 after challenge infection; Groups 2 and 3 were the controls for sensitization and challenge infection, respectively.
† Day 45, day 0 post-reinoculation (p.r.i.).
‡ ML burden in pepsin-digested tissues and counting the larvae in triplicate samples/rat. Data are mean ± 1 S.D. from numbers of rats included in each group.
§ L_1, infective-stage larvae of *T. spiralis*.
¶ No significantly different number of ML compared to either group 2 (sensitization control) and group 3 (challenge control) rats (Unpaired, two-tailed student's *t*-test, $P > 0{\cdot}05$).

reinfection studies. These included (1) failure of any challenge L_1 to develop into NBL-producing adults, or (2) immediate trapping and elimination of NBL. The present study was performed to investigate the real state of affairs operative in our rat system. We measured HSP25, HSP60 and HSP70 levels in rat intestine, MLN, heart and lungs during first and second *T. spiralis* infection using Western blotting analyses with the following aims. Those were, firstly, to determine reinfection-induced altered intestinal damage and MLN trapping of NBL (if any) and, secondly, to confirm prevention of shock responses (also) in reinfected rat heart and lungs. We demonstrated that, despite complete removal of the challenge ML dose within 7 days, reinfected rats had MLN tissues presenting signs of NBL trapping-mediated damage. We also demonstrated that, unexpectedly, lasting shock responses mediated by, as yet, unknown factors occurred in reinfected rat heart tissues.

MATERIALS AND METHODS

Antibodies

Mouse anti-HSP60 (clones LK-1 and LK-2) and anti-HSP70 (clone BRM-22) were from Sigma. Rabbit antiserum to recombinant mouse HSP25 (SPA801) was from Stressgen. Peroxidase-conjugated goat anti-mouse and anti-rabbit Abs were from Sigma.

Parasite, infection protocol and collection of experimental tissues

The preparation of infective-stage *T. spiralis* larvae (L_1), the housing conditions, the health status of test rats post-inoculation and euthanasia applied in these studies have been reported in detail (Martínez *et al.* 1999*a*).

Female Wistar rats, weighing approximately 200 g, were inoculated orally on day 0 with either 1 ml of non-pyrogenic isotonic solution (NPIS) only (uninfected controls; $n = 7$) or NPIS containing 6000 L_1 (primary-infected rats; $n = 33$). Forty-five days thereafter (i.e. day 0 for reinfection experiments), 2 uninfected control rats received a *T. spiralis* infection (secondary infection controls), 3 primary-infected rats were given NPIS only (reinfection 'blanks') whereas 15 others were given a secondary *T. spiralis* infection. At days 1, 7, 14, 20 and 27 p.i., or post-reinfection (p.r.i.), 3 infected rats together with 1 uninfected control, or 3 reinfected rats, respectively, were killed. At days 1, 14 and 27 p.r.i. time-point 1 reinfection 'blank' was also killed for obtaining time-matched normal background levels of our test HSPs. Immediately after euthanasia, small intestine, MLN, heart and lung of each rat were removed. These organs, with the exception of days 1, 7 and 14 p.i. or p.r.i. small intestines (which were rid first of worms) were then rapidly rinsed in saline, drained and stored in liquid nitrogen until processed.

Worms embedded in the small intestinal mucus were collected and counted after the method of Denham & Martínez Fernandez (1970). Estimates of ML burdens were obtained following pepsin digestion of the skinned and eviscerated remains of each of 3 rats killed at days 20 and 27 p.i. or p.r.i. Infectivity of our batch of challenge L_1 was verified by the finding of substantial numbers of encysted ML in our 2 secondary infection controls at the end of the experiment (see Table 1).

Processing of rat tissues

The procedures utilized for obtaining soluble rat tissue preparations for analysis of HSP production have been reported in detail (Martínez *et al.* 1999*a*). Specimens were analysed for protein content by the Bradford procedure (Bio-Rad) and stored in liquid nitrogen until needed.

Immunoblot assay for HSPs 25, 60 and 70

Rat tissue samples were assayed for HSP content by immunoblot assay as previously described in detail (Martínez *et al.* 1999*a*, *b*). Briefly, equal amounts of protein were electrophoresed through sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing and denaturing conditions, electroblotted onto PVDF (polyvinylidene fluoride) membranes, and probed using anti-HSP25, anti-HSP60 or anti-HSP70 as primary antibody and peroxidase-conjugated secondary antibody. This was

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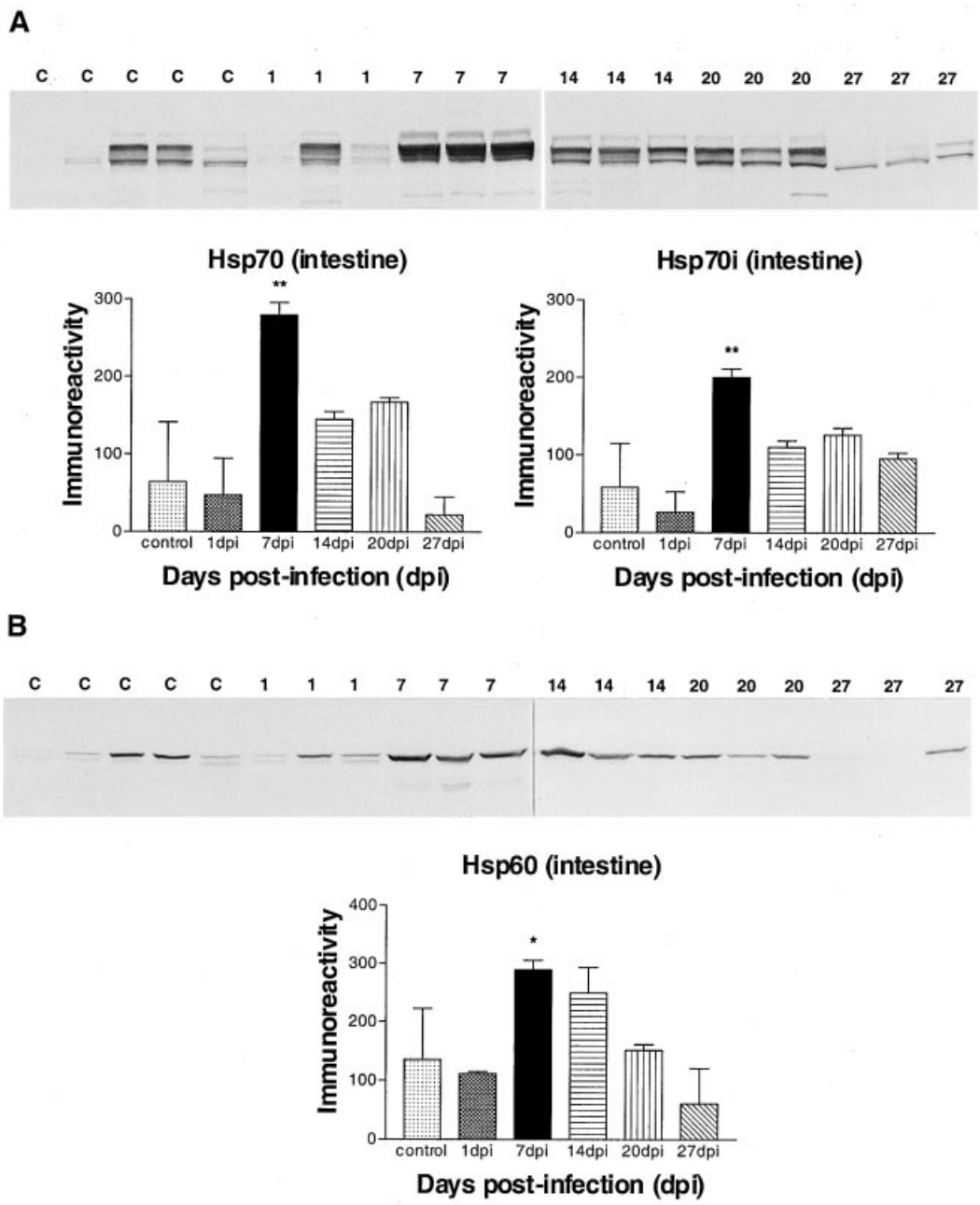

Fig. 1. Levels of HSP70/HSP70i (A) and HSP60 (B) in intestines during a primary *Trichinella spiralis* infection. Specimens C were from uninfected control rats. Bars represent the mean ± S.D. from 3 or 5 individual samples. Upper part of panels illustrates the representative blot used to generate the data shown in the figure. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

followed by addition of H_2O_2 and the substrate 3,3′ diaminobenzidine for visualization of immune reactions.

Densitometric and statistical analysis

HSP levels in each test tissue, quantitated by the relative optical densities (O.D.) of the corresponding images on immunoblots, were determined using an image scanning densitometer and associated software (Program Quantity One, Bio-Rad). Statistical analysis of the mean differences in immunoreactivity (i.e. relative O.D.) between samples from control rats and those from infected rats were analysed by the Dunnet test. A probability value of 0·05 (95% confidence) was used to test for significance.

Linearity of Western blot test results

Prior to this study experiments were carried out to demonstrate linearity of immunoblot results at our testing conditions. Recovery of each HSP by its HSP-specific antibody to be employed in the Western blot was assessed by assaying rat intestine, MLN, heart and lung specimens with varying amounts of proteins. Evaluation of test results

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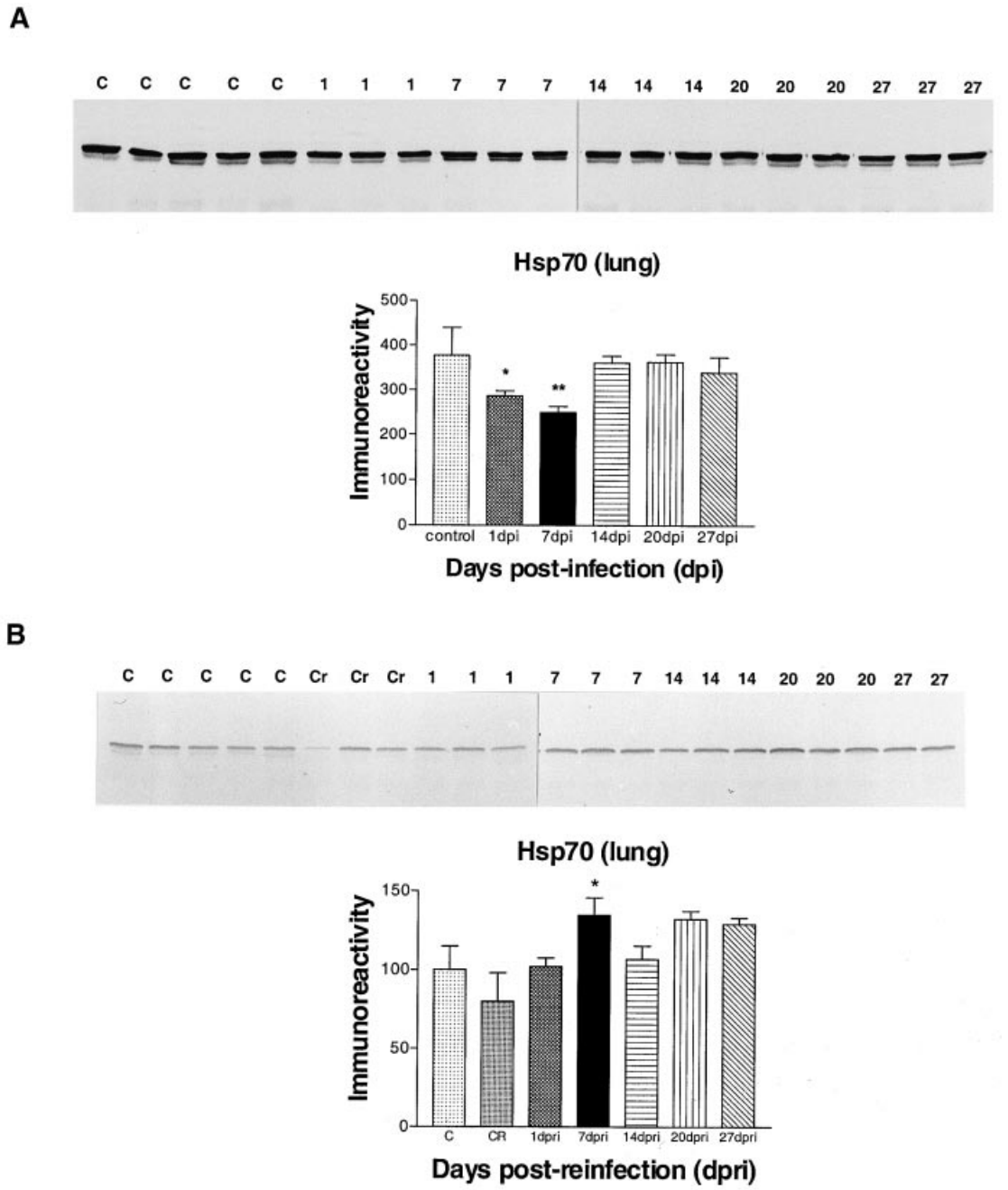

Fig. 2. HSP70 expression levels in lungs during a primary (A) or secondary (B) *Trichinella spiralis* infection. Specimens C were from uninfected control rats and CR were from (control) rats with a primary infection only. Bars represent the mean ± S.D. from 3 or 5 individual samples. Upper part of panels illustrates the representative blot used to generate the data shown in the figure. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

obtained by regression analysis consistently produced a very high correlation ($r > 0{\cdot}95$) between observed immunoreactivity and amount of protein applied in the test.

RESULTS

In intestine from once-only infected rats increased expression of HSP60 and HSP70 began between days 2 and 7 p.i. with significantly increased levels evident on day 7 p.i. and thereafter returned to normal control levels (Fig. 1A, B). Infection caused the induction of a protein (termed HSP70i) migrating slightly faster than HSP70 during electrophoresis and reacting with our anti-HSP70 antibody. Interestingly, HSP70i production kinetics and its level fluctuation dynamics seemed to exactly follow those observed for HSP70 (Fig. 1A), suggesting a unitary regulation of rat HSP70 and HSP70i production. In contrast, HSP60 and HSP70 levels in reinfected rats' intestines never differed significantly from those in controls; the former though, had substantially more HSP60 at day 7 p.r.i., this

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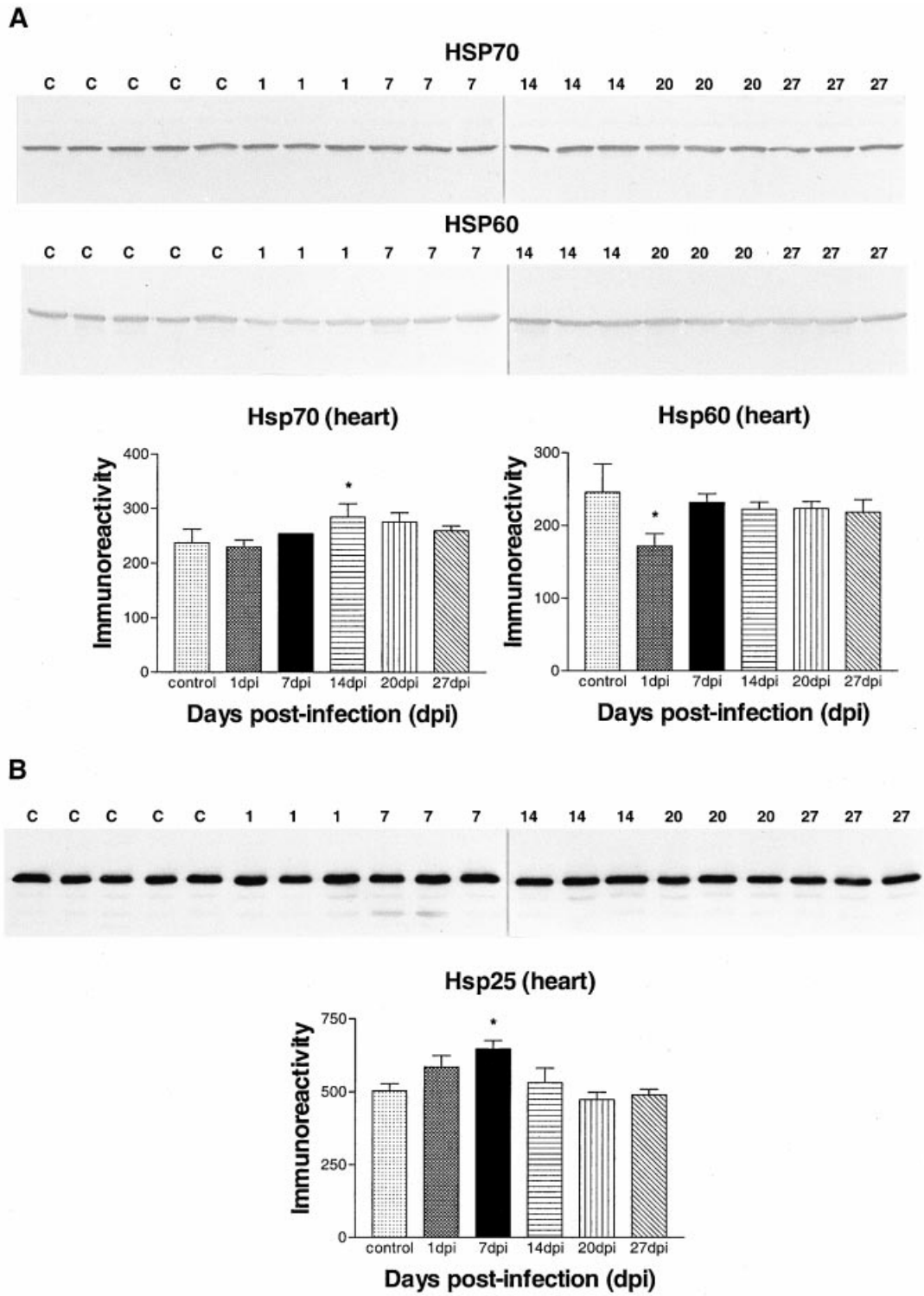

Fig. 3. Levels of HSP70 or HSP60 (A) and HSP25 (B) in heart tissues during a primary *Trichinella spiralis* infection. Specimens C were from uninfected control rats. Bars represent the mean ± S.D. from 3 or 5 individual samples. Upper part of panels illustrates the representative blot used to generate the data shown in the figure. $*P < 0{\cdot}05$.

thereafter returning to normality (data not shown). The rabbit polyclonal antibody, when used to detect HSP25 in intestinal specimens, consistently failed to do so.

Evaluation of HSP25, HSP60 and HSP70 levels in lung tissue specimens from once-only infected and reinfected rats was performed. Levels of the former 2 HSPs differed at no time-point from those in controls and are, therefore, not shown. Analyses of lung tissues from once-only infected rats (Fig. 2A) revealed that HSP70 levels were significantly reduced when compared with those in control rats at days 1 and 7 p.i. but attained normal levels thereafter. In reinfected rats, lung HSP70 levels began to

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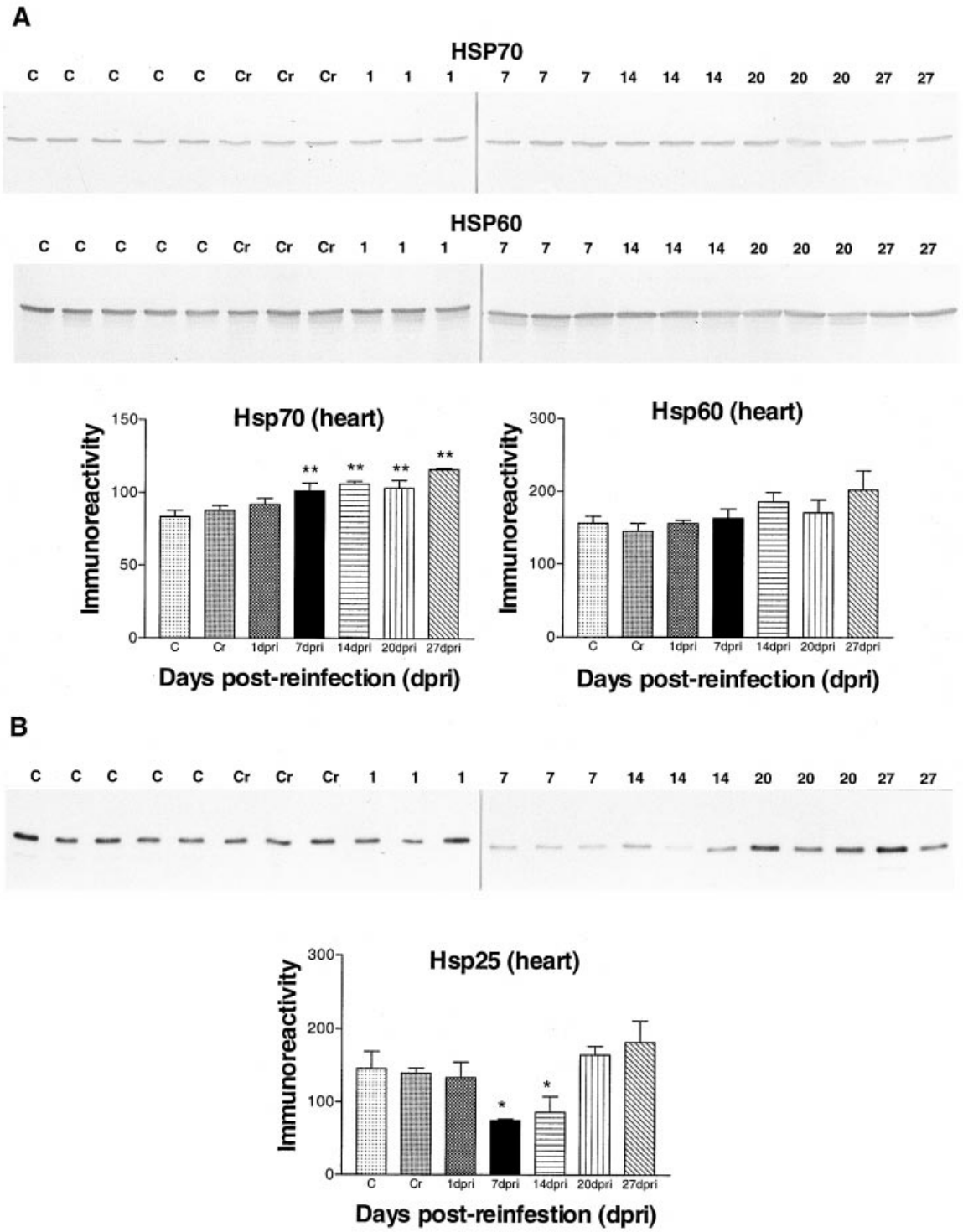

Fig. 4. Levels of HSP70 or HSP60 (A) and of HSP25 (B) in heart tissues during a secondary *Trichinella spiralis* infection. Specimens C were from uninfected control rats and Cr were from (control) rats with a primary infection only. Bars represent the mean ± S.D. from 3 or 5 individual samples. Upper part of panels illustrates the representative blot used to generate the data shown in the figure. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

increase somewhere between days 2 and 7 p.i. with significantly increased levels versus controls evident at day 7 p.r.i., but reached normal levels by day 14 p.r.i. (Fig. 2B).

As regards test HSP levels in heart tissue specimens from once-only infected rats the significantly (i) increased HSP25 and HSP70 levels at days 7 and 14 p.i., respectively and (ii) decreased HSP60 level at day 1 p.i., compared to controls, deserve mention (Fig. 3A, B). In reinfected rats, significantly increased heart tissue HSP70 levels compared to controls were evident from day 7 p.r.i. up to day 27 p.r.i. (Fig. 4A); HSP25 levels were significantly reduced from days 7 to 14 p.r.i. and thereafter

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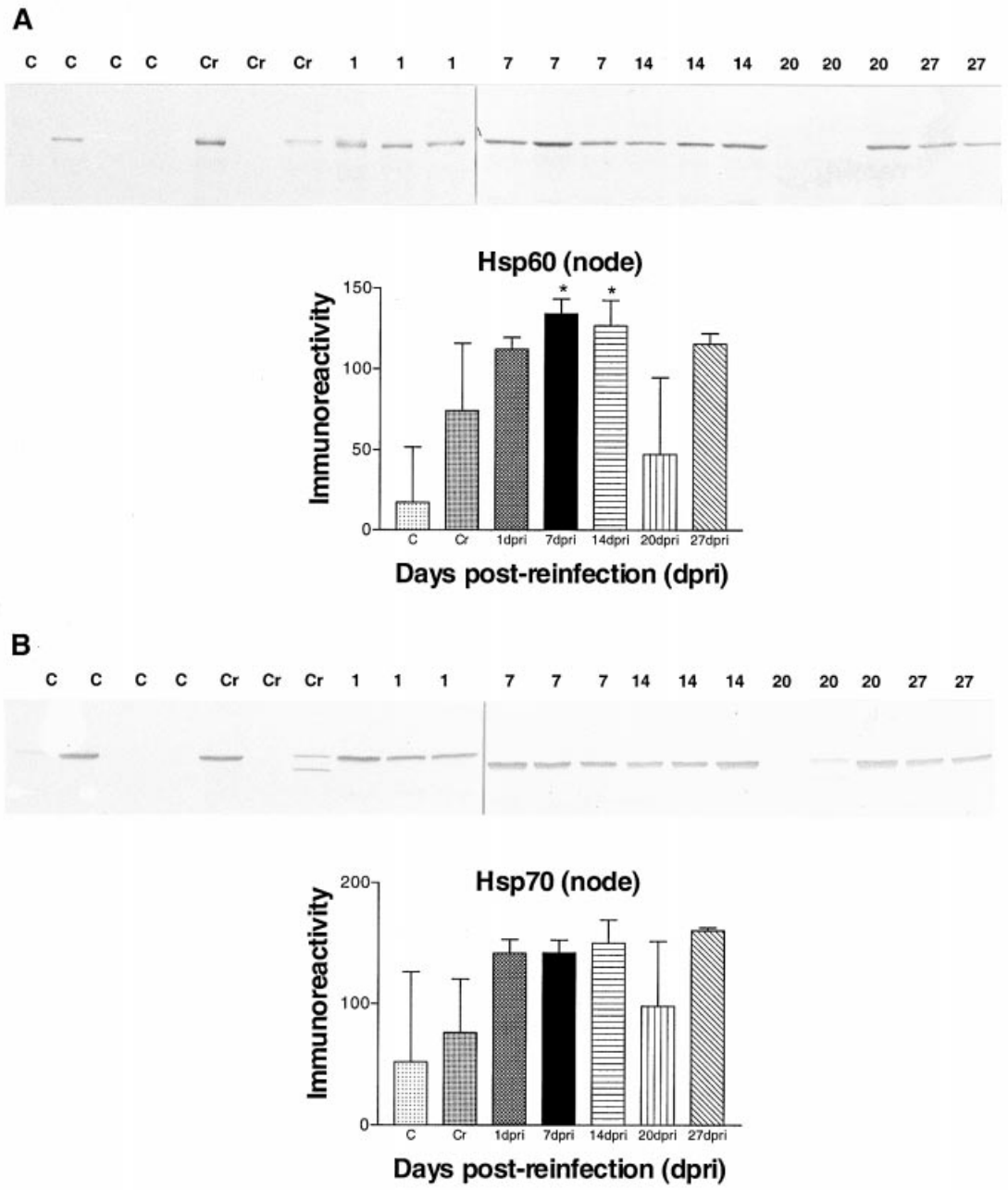

Fig. 5. Levels of HSP60 (A) and HSP70 (B) in mesenteric lymph nodes during a secondary *Trichinella spiralis* infection. Specimens C were from uninfected control rats and Cr were from (control) rats with a primary infection only. Bars represent the mean ± S.D. from 3 or 5 individual samples. Upper part of panels illustrates the representative blot used to generate the data shown in the figure. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

attained normal levels (Fig. 4B). HSP60 levels in reinfected heart tissues did not differ from those in controls at any time during the course of the study (Fig. 4A).

In MLN from primary infected rats there was no altered expression of HSP60 and HSP70 (not shown), suggesting that no *T. spiralis* larval stage (adults, NBL) affected detectable stress therein within the time-course of the study. In contrast, HSP60, but not HSP70, was found to be produced at significantly higher levels than in control MLN at days 7 and 14 p.r.i. (Fig. 5A, B). It was of note, that also in MLN specimens the polyclonal antibody SPA801 consistently failed to detect HSP25.

Anti-NBL immunity in rats exposed to test L_1 was also examined by counting ML after digesting their tissues with pepsin (Table 1). Great numbers of larvae were recovered 27 days (group 3) and 65 + 72 days (group 2) after primary infection, indicating good function of challenge L_1. The number of larvae recovered after secondary infection was comparable ($P > 0{\cdot}05$) to that from rats kept without secondary infection (group 2). We thus conclude that in our reinfected rat model effective anti-*T. spiralis* mech-

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anisms were operative, these preventing any significantly increased ML burden to become evident.

DISCUSSION

Since ML must develop into (fertile) adults for trichinosis to occur, their ability to maintain a long enough presence in the host's gut is a major 'virulence' determinant. This ability is almost lost in immunized rats (Bullick, Russell & Castro, 1984); moreover, immune mechanisms here affect rapid trapping and clearing of NBL (Wang & Bell, 1987). Accordingly, we noted increased HSP production in 4 rat organs during primary (Martínez *et al*. 1999*a*, *b*), but not secondary *T. spiralis* infections (Martínez *et al*. 1999*c*). However, while informative, the latter work could not exactly pinpoint the extrinsic process underlying the observed phenomenon. An obvious drawback to the study is that intestinal parasite burden was evaluated at days 1 and 7 p.r.i. and not at an intervening time (e.g. day 3 p.r.i.). So, we here set out to tackle the above problem by examining both infection and reinfection-induced stress responses in (i) intestines and MLN ('direct-contact' organs) and (ii) heart and lung tissues (which we assumed would give results as noted in previous studies). Although, some results were as expected, many others were not. Three new and interesting observations were made.

Firstly, in addition to finding, in intestines, increased HSP60 and HSP70 levels at day 7 p.i., the time of maximum NBL production (Harley & Gallicchico, 1971), commencement of worm expulsion (Bell, 1998) and appearance of signs of enteric discomfort (Capó & Despommier, 1996), we observed the induction of an HSP70i molecule. The function of this HSP70i is unknown but our data indicate a strong relationship with HSP70, as its production, followed that of HSP70. In contrast, reinfection caused neither HSP70i production, nor significantly altered levels of HSP60 or HSP70, suggesting that above elements affecting intestinal shock responses were not operative in the immune rat. Careful scrutiny of test results, however, warns against such a conclusion. Like their once-only infected counterparts reinfected rats had intestinal HSP60 levels that only after day 7 dropped to normality. Increased intestinal HSP production between days 1 and 7 p.(r.)i., thus, seems to be a very general feature of (re)infected rats and is due to adult worm activities. Critical evidence for incomplete resistance to *T. spiralis* through anti-L_1 immunity (rapid expulsion) has been reported using challenge-infected suckling rats; such rats have appreciable numbers of ML (Appleton & McGregor, 1984). Because the present study included no evaluation between days 1 and 7 p.r.i., we cannot directly address this question. Overall findings (see below also), nevertheless, justify us concluding that, also in our immune adult rat system, challenge L_1 escape immune expulsion and develop into NBL-producing adults.

Secondly, we observed different responses in MLN obtained from either once-only infected or reinfected rats. While the former obviously had greater numbers of NBL entering into their circulation, HSP levels differing from uninfected controls were never noted, suggesting an insignificant role for MLN in NBL trapping during primary infection. In contrast, reinfection of rats resulted in significantly increased HSP60 levels at day 7 p.r.i., this situation persisting for at least 1 week. This observation may aid in our further understanding the differential dealing with a *T. spiralis* infection by either the naive or the immune rat. It would seem that, following reinfection, NBL produced would be transported to trapping centres and destroyed. The latter process entails release (by killer cells) of, for example, H_2O_2 (Butterworth, Vadas & David, 1981) and cytokines, i.e. molecules directly and indirectly causing stress injury to surrounding tissues (Polla, Perin & Pizurki, 1993). Secondary infections of rats with *T. spiralis* are associated with increased NBL destruction (Wang & Bell, 1987) and MNL seem to be, as we show here, an important organ in which larval trapping and subsequent immobilization occurs. Indeed preliminary histopathological studies in our laboratory (unpublished) indicate clear MLN trapping of NBL within eosinophilic granulomas. Our observations justify the construction of a new and larger study to further explore this question.

Thirdly, our including comparison of (re)infection-mediated HSP expression levels in 2 additional tissues enabled us to now determine that reinoculation may affect increased HS responses in some rat organs. A major finding in the present study is that heart tissue specimens from reinfected rats contained significantly increased HSP70 levels evident from day 7 up to day 27 p.r.i. This observation may have important clinical implications. It would seem that, in the immunized host, reinoculation would trigger off mechanisms (immune and/or non-immune ones) that would induce relatively rapid and prolonged 'stress' to the host's heart tissue. The exact nature of these 'triggers' is unknown. Our findings justify additional larger studies, which should include histo(patho)logical examinations of heart tissues, to elucidate the process underlying the here observed heart stress response associated with *T. spiralis* (re)inoculation.

In conclusion, this study provides evidence that in immune rats, reinoculation with *T. spiralis* ML evokes increased and prolonged HS responses in the animals' heart and MLN. Although straightforward interpretation of our data seems to point to immune destruction of NBL as the most likely (if not the only) initiator of the observed phenomenon, another study will be necessary to prove this contention and

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evaluate pathological consequences. Given the similarities between human, rat (and e.g. pig) trichinosis the results of such studies may be of great importance in clinical practice.

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