Heat shock response of *Trichinella spiralis* and *T. pseudospiralis*

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(*Received 28 January 1995; revised 24 May 1995; accepted 24 May 1995*)

SUMMARY

Heat shock proteins (HSPs) were documented for the first time in both somatic extracts and excretory/secretory (ES) products of the infective-stage larvae of *Trichinella spiralis* and *T. pseudospiralis*. Larvae recovered from muscles of infected mice were heat shocked at 37, 40, 43 and 45 °C in RPMI 1640 medium containing L-[^{35}S]methionine. Somatic extracts and ES products of heat-shocked worms were then analysed by SDS–PAGE, autoradiography and laser densitometry. Prominent bands of HSPs were observed at 43 °C which is the optimal heat shock temperature. The major HSPs in somatic extracts of *T. spiralis* were 20, 47, 50, 70, 80 and 86 kDa. When the temperature was increased from 37 to 43 °C, the greatest increase in absorbance was observed in HSPs 70 and 86. *In vitro* translation of mRNA in a nuclease-treated rabbit reticulocyte lysate system showed an increase in the synthesis of the 80 kDa protein. This suggests that the production of HSP 80 is regulated at the transcriptional level. The major HSPs in the ES products were 11, 45, 53 and 64 kDa. In *T. pseudospiralis*, the major HSPs in the somatic extracts were 20, 26, 31, 50, 53, 70, 80 and 86 kDa, and in the ES products, 11, 35, 37, 41 and 64 kDa.

Key words: *Trichinella spiralis*, *Trichinella pseudospiralis*, nematode, heat shock proteins, excretory/secretory products.

INTRODUCTION

Trichinella spiralis is an unique monoxenous nematode which lacks a free-living dispersal stage. The infection is transmitted by direct ingestion of meat containing the first-stage larvae. The larvae can develop in striated muscles of all mammals where they reorganize the host cells into 'nurse cell complexes'. Each complex consists of a cytoplasmic region with hypertrophic myonuclei, small cells, endoplasmic reticulum, secretory vesicles, mitochondria and a cavity containing 1–4 worms.

The molecular mechanism of host cell reorganization by intracellular nematodes is unknown. However, our recent immunocytochemical studies (Lee *et al.* 1991; Ko *et al.* 1994) showed that excretory/secretory (ES) molecules from the infective-stage larvae of *T. spiralis* may directly interact with myocytes at the nuclear level. Ko *et al.* (1994) postulated that the invasion of muscles by trichinellids elicited two distinct events i.e. a general degenerative/regenerative response of muscles and a specific change in genomic expression of myonuclei. The two events are probably mediated by different effector molecules which initiate the infectious process and modulate the host environment. One event leads to the dissolution of myofibres, hypertrophy of myonuclei, myotube formation, angiogenesis etc. The other event involves the translocation of molecules into the myonuclei to alter and regulate genomic expression.

To test the above hypothesis, it is, however, necessary first to analyse the composition of ES products and somatic extracts of *Trichinella*. One immediate objective is to ascertain the presence of proteins which may be involved in molecular translocation. Since heat shock proteins (HSPs) are known to fold molecules for translocation into the nucleus, mitochondrion or microsome (Pelham, 1988), a study was initiated on the hitherto unknown heat shock response in trichinellosis. The initial phase of the study was to document the presence of HSPs in both somatic extracts and ES products of the infective-stage larva of *T. spiralis*. An *in vitro* translation of mRNA from heat-shocked larvae was also undertaken to determine whether the HSPs are regulated at the transcriptional or translational level.

The heat shock response of a closely related species, *Trichinella pseudospiralis*, was also studied. Unlike *T. spiralis*, this trichinellid can develop in both birds and mammals. Therefore, different HSPs may be implicated in such an extraordinary parasitic adaptation.

MATERIALS AND METHODS

Infective-stage larvae were used for the present study because they yield more protein than the newborn worms. But most importantly, the former larvae have also been shown to be capable of forming nurse cells (Ko *et al.* 1994).

The larvae were obtained from muscles of experimentally infected ICR mice by the standard pepsin digestion method. After recovery, the larvae were washed 5 times in 0·8% saline before being transferred to methionine-free RPMI 1640 medium (Sigma) under axenic conditions. The medium, which was supplemented with L-glutamine, L-leucine and L-lysine (Sigma) and HEPES (Flow Lab), was sterilized by filtration through a 0·22 μm pore size membrane (Millipore). The worms were heat shocked and radio-isotope labelled.

Radio-isotope labelling

Approximately 2500 larvae were placed into a sterile 17 × 100 mm round-bottomed polypropylene tube (Falcon) containing 0·3 ml of the above medium which was pre-warmed to 37 °C. They were then metabolically labelled with L-[^{35}S]methionine (200 μCi/ml, specific activity > 1000 Ci/mmol, Amersham) in a shaking water-bath (Dubnoff, Precision) at 37, 40, 43 and 45 °C. The labelling period was relatively prolonged (2–4 h) because the nematode cuticle prevents fast uptake of methionine. The culture medium containing the labelled ES products was removed and stored.

To study the kinetics of synthesis of HSPs, the worms were equilibrated at 43 °C for 30 min, 1 h, 2 h or 4 h, before adding the label.

The worms were examined regularly under a stereomicroscope to ensure that they remained alive and active during heat shock treatment and radio-isotope labelling.

The larvae were washed twice in phosphate-buffered saline (pH 7·4) to remove any unincorporated label. They were then disrupted by a sonicator equipped with a microtip and pulsar cycle (Heat Systems Ultrasonics, W-385). After sonication, the sample was allowed to extract for 4 h at 4 °C. Tissue debris was removed by centrifugation for 30 min at 4390 ***g*** (Hermle, Z 231M) and 4 °C. The supernatant fraction was aliquoted and stored at −70 °C.

The protein concentration of the somatic extracts and ES products was determined using a protein assay kit (BioRad).

Autoradiography

The somatic extracts and ES products were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) in a Mini-Protean II cell (BioRad), following the method of Ko & Yeung (1991). A 4% stacking and 7–10% separating gel was used. Samples (4 μg/well) were run under reducing conditions and the gel was then stained with Coomassie Brilliant Blue R250. After destaining and rinsing in distilled water, the gel was blotted dry on chromatographic papers (3 mm Chr, Whatman) before being placed in a slab gel dryer (Hoefer SE 1160). The dried gel was exposed to a X-ray film (Kodak, X-OMAT AR 5) in a stainless steel cassette (Sigma). For somatic extracts, the gel was exposed for 10 days whereas for ES products it was exposed for 15 days. A longer exposure was required for the latter because the quantity of labelled ES proteins was smaller. The exposure time of the *in vitro* translation gel was 14–16 h.

Besides autoradiography, fluorography was also employed to analyse the gels. However, since the results of two methods were similar, only the autoradiographic data are given in this paper.

Extraction and isolation of mRNA

Sterile disposable plastic-ware, free of ribonucleases, was used for extracting RNA.

After recovery from muscles, the larvae were washed 7 times in saline before being transferred to polypropylene tubes (Falcon). Each tube contained 2 ml of RPMI medium and 0·4 ml of worms. They were heat shocked for 2 h at 43 °C in a water-bath. Worms incubated at 37 °C served as the negative control. After heat shock, the worms were frozen in liquid nitrogen and stored at −70 °C until extraction.

A Quickprep mRNA purification kit (Pharmacia) was used to extract RNA. A 0·5 g sample of frozen larvae was suspended in 1·5 ml of extraction buffer containing guanidinium thiocyanate and *N*-lauroyl sarcosine and homogenized 7 times (15 sec each) at 24000 r.p.m. in a icebath using a polytron homogenizer (Janke & Kunke, Ultra-Turrax T25). Then 3 ml of elution buffer containing 10 mM Tris–HCl (pH 7·4) and 1 mM EDTA was added to the homogenate and mixed thoroughly. The diluted homogenate was then centrifuged for 10 min at 10000 r.p.m. (Beckman J2-HS JA 20-1 rotor). The supernatant fraction contained the total RNA.

Three ml of the supernatant containing the total RNA was transferred onto the surface of the resin in an oligo(dt)-cellulose spun column. The resin and supernatant were mixed gently for 10 min by inverting the column. Poly (A)$^+$ mRNA was allowed to bind to oligo(dt)-cellulose. To separate the resin from the suspension, the column was centrifuged for 2 min at 1300 r.p.m. (Beckman GPR) and room temperature. The suspension was washed by centrifugation, first 3 times using a high-salt buffer (10 mM Tris–HCl pH 7·4, 1 mM EDTA, 0·5 M NaCl) and then twice with a low-salt buffer (NaCl reduced to 0·1 M).

The bound poly (A)$^+$RNA was eluted with an elution buffer (10 mM Tris–HCl, pH 7·4, 1 mM EDTA) which was pre-warmed to 65 °C. The elution was carried out in 3 successive washings by centrifugation. The absorbance of the eluate was

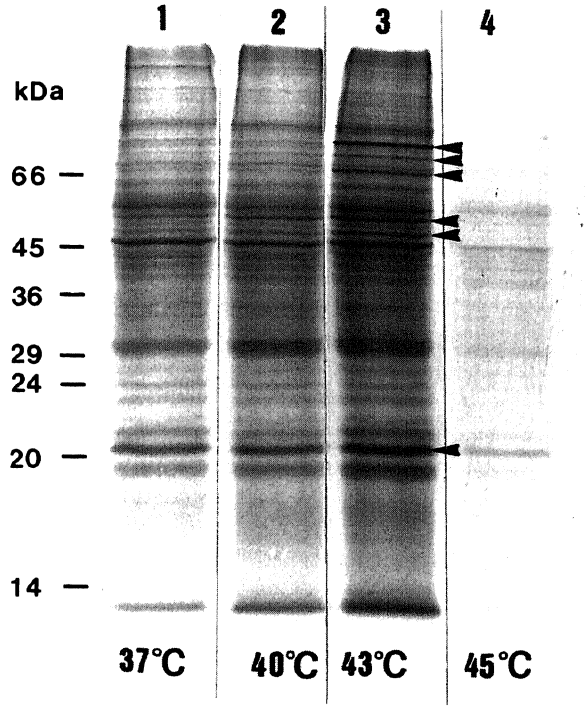

Fig. 1. Autoradiogram showing SDS–PAGE profile of [^{35}S]methionine-labelled somatic extracts of infective-stage larvae of *Trichinella spiralis* given heat-shock treatment and radio-isotope labelling for 2 h at various temperatures. Note the presence of more prominent bands in Lane 3. Arrows indicate major heat shock proteins of 20, 47, 50, 70, 80 and 86 kDa.

read at 260 nm in a spectrophotometer (Hitachi U-2000).The purity of the mRNA was ascertained by the OD_{260}/OD_{280} ratio of sample. Only samples with a ratio greater than 2 were used. A ratio less than 2 indicates the presence of proteins.

Diluted mRNA (0·5 ml) was precipitated with 10 μl of 1 % glycogen solution, 1/10 vol. of 2·5 M potassium acetate (pH 5·0) and 2 vols of 95 % ethanol which was pre-cooled to −20 °C. The sample was kept for 2 h at −20 °C. The precipitated RNA was pelleted by centrifugation for 1 h at 340 r.p.m. and 4 °C (Beckman GPR). The air-dried mRNA pellet was redissolved in an appropriate volume of diethyl-pyrocarbonate (DEPC, Sigma)-treated water.

In vitro *translation*

In vitro translation of poly $(A)^+$ mRNA was performed using a nuclease-treated rabbit reticulocyte lysate kit (Promega).

After heating the mRNA sample for 10 min in a water-bath at 67 °C, it was cooled immediately in an ice-bath. Reagents were then added into a 0·5 ml vol. polypropylene microcentrifuge tube immersed in ice in the following order: 35 μl of rabbit reticulocyte, 7 μl of nuclease-free water, 1 μl of RNasin ribonuclease inhibitor, 1 μl of 1 mM amino acid mixture (minus methionine), 4 μl of [^{35}S]methionine (specific activity > 1000 Ci/mmol) (Amersham), 2 μl of mRNA from heat-shocked or normal larvae at 200 μg/ml of water.

The final volume of the reticulocyte translation reaction was made up to 50 μl. The sample was incubated at 30 °C in a shaking water-bath (Dubnoff, Precision) for 100 min.

The cell-free translation products were analysed by SDS–PAGE as described previously. Luciferase RNA and Brome Mosiac Virus (BMV) RNA served as the positive control. For the negative control, the RNA substrate was replaced by DEPC-treated water.

Densitometry

The absorbance of the bands in the autoradiograms was quantified and compared using a laser densitometer (UltroScan XL, Pharmacia) with a wave length of 633 nm. The base line of the scan was set as the average of 16 lowest points.

RESULTS

Effect of temperature on protein synthesis in T. spiralis

Crude somatic extracts. The autoradiogram of worms isotope-labelled at 37 °C showed more than 31 bands ranging from 19 to 110 kDa (Fig. 1). A similar profile was also observed for the 40 °C sample. However, at 43 °C, bands of 20, 47, 50, 70, 80 and 86 kDa were wider and intenser than those from the previous two temperatures. The increase in absorbance appeared to be most prominent at 86 (HSP 86) and 70 (HSP 70) kDa. At 45 °C, in contrast, only faint bands ranging from 20 to 90 kDa were observed. Of these, bands of 53, 45 and 20 kDa were the most intense.

The densitometric data showed that there were substantial increases in the peaks of all HSP bands at 43 °C (Fig. 2). The absorbance of HSPs at 43 °C was 47–120 % higher than those at 37 °C. The greatest increase in absorbance was shown by HSP 86, followed by HSP 70.

Equilibration of worms for 30 min to 4 h at 43 °C prior to the addition of the isotope label failed to produce any significant difference in the intensity of HSP bands.

Monoclonal antibody against mammalian HSP 70 isolated from bovine brain (Sigma) was applied to sections of heat-shocked larvae using the indirect immunoperoxidase method. Positive reactions were observed in the muscle layer and in the stichocytes of worms.

ES products. At 40 °C, 3 weak bands of 45, 53 and 64 kDa were observed in the autoradiogram. At 43 °C, at least 21 bands were resolved but the most prominent ones were HSPs 45, 53 and 64 kDa (Fig.

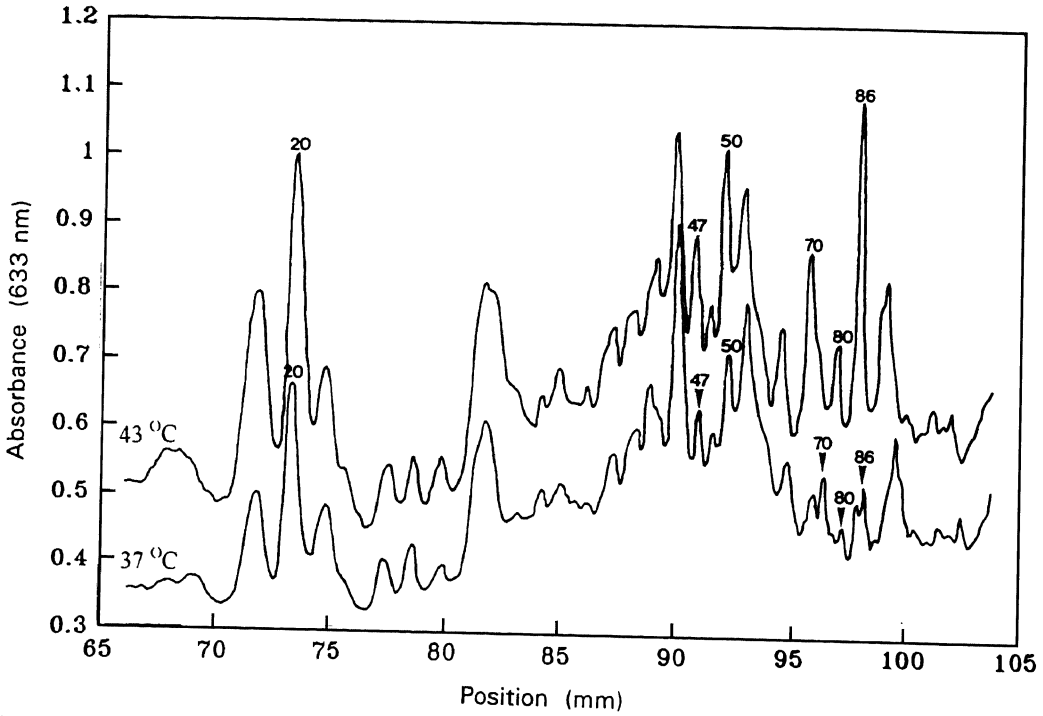

Fig. 2. Densitometric data comparing absorbance of bands in autoradiogram of methionine-labelled somatic extracts of infective-stage larvae of *Trichinella spiralis* given heat-shock treatment and radio-isotope labelling for 2 h at 37 and 43 °C. Base line of scan represents average of 16 lowest points. Number on peak indicates molecular mass.

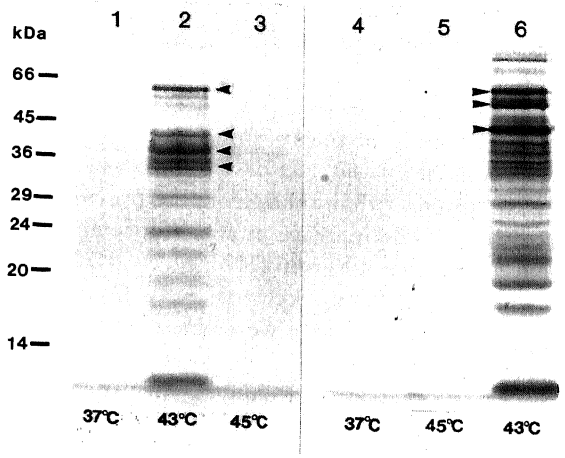

Fig. 3. Autoradiogram comparing SDS–PAGE profile of [^{35}S]methionine-labelled excretory–secretory products of infective-stage larvae of *Trichinella pseudospiralis* (Lanes 1–3) and *T. spiralis* (Lanes 4–6) given heat-shock treatment and radio-isotope labelled for 4 h at various temperatures. Arrows indicate major heat shock proteins.

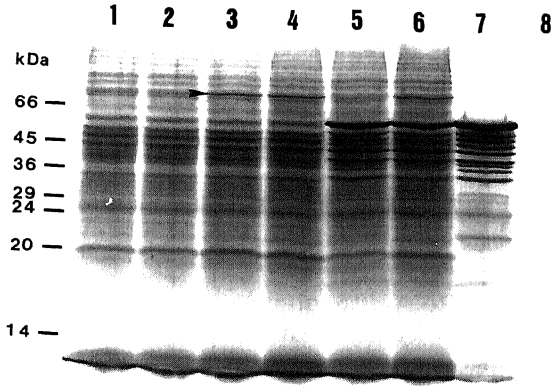

Fig. 4. Autoradiogram showing SDS–PAGE profile of *in vitro* translation products using 1 or 2 μg mRNA from infective-stage larvae of *Trichinella spiralis* maintained for 2 h at 37 or 43 °C. Lanes 1 and 2 – worms at 37 °C, using 1 and 2 μg control mRNA as substrate. Lanes 3 and 4 – worms at 43 °C, using 1 and 2 μg sample mRNA as substrate. Arrow in Lane 3 indicates HSP 80. Lane 5 – control mRNA (2 μg) mixed with luciferase control RNA. Lane 6 – sample mRNA (2 μg) mixed with luciferase control RNA. Lane 7 – luciferase control RNA. Lane 8 – non-exogenous mRNA blank.

3). All three bands were substantially more intense than those from 40 °C. No bands were observed at 37 and 45 °C.

A significant increase in the intensity of HSPs 64 and 11 was noted when the equilibration time of worms at 43 °C was increased from 30 min to 4 h.

In vitro *translation*

Between 38 and 56 μg of mRNA were isolated from 0·5 g of infective-stage larvae of *T. spiralis* by the QuickPrep mRNA Purification Kit (Pharmacia). In the control experiment at 37 °C, proteins translated were demonstrated in the mixture of luciferase control RNA, worm RNA and in the BMV RNA positive control.

Fig. 4 compares the autoradiograms of the *in vitro* translation products of worm mRNA incubated at 37 and 43 °C, using 1 or 2 μg of mRNA as substrate. At

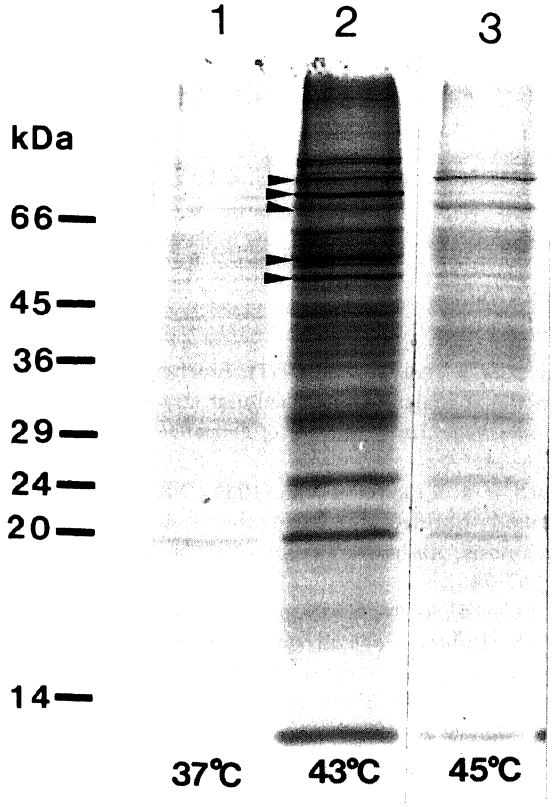

Fig. 5. Autoradiogram showing SDS–PAGE profile of [^{35}S]methionine-labelled somatic extracts of infective-stage larvae of *Trichinella pseudospiralis* given heat-shock treatment and radio-isotope labelled for 4 h at various temperatures. Arrows indicate major heat shock proteins.

least 30 polypeptides, ranging from 20 to 110 kDa, were present in the translation products of worm mRNA. However, at 43 °C, a prominent band of 80 kDa was observed when either worm mRNA or a mixture of worm RNA and luciferase control RNA was used as substrate.

The densitometric data showed that at 43 °C the absorbance of the 80 kDa band was about 30% greater than that of 37 °C.

Effect of temperature on protein synthesis in T. pseudospiralis

Crude somatic extracts. At 37 or 45 °C, only faint bands were present in the autoradiogram. However, at 43 °C, darker bands were observed. The major HSPs were 20, 26, 31, 50, 53, 70, 80 and 86 kDa (Fig. 5).

ES products. At 37 or 45 °C, only 2–3 very faint bands were present in the autoradiogram. However, at 43 °C at least 14 bands were observed. The major HSPs were 35, 37, 41 and 64 kDa (Fig. 3).

DISCUSSION

The present study documents for the first time the presence of HSPs in both somatic extracts and ES products of trichinellid nematodes. The densitometric data clearly demonstrate that there was a substantial increase in HSPs when the worms were incubated at 43 °C which is the optimal heat-shock temperature. Data of the *in vitro* translation of mRNA show conclusively that the production of one of the HSPs (HSP 80) is regulated at the transcriptional level.

Seven of the major HSPS of *T. spiralis* and *T. pseudospiralis* share the same molecular masses. In somatic worm extracts, there are 5 common HSPs (HSP 20, 50, 70, 80 and 86 kDa) whereas in the ES products, there are only 2 (11 and 64 kDa). The difference in the HSP profile between the two species probably reflects the difference in their respective mode of parasitism, in particular, host specificity and host interaction. Non-conserved stress proteins are probably involved in the more versatile adaptation of *T. pseudospiralis* which can undergo growth and development in either mammals or birds and without encapsulation.

The presence of HSPs in ES products of nematodes has not been reported previously. In the present study, the labelled proteins detected in ES products of *Trichinella* are regarded as HSPs because their production was entirely temperature dependent. There was also a significant increase in production when the equilibration time of worms prior to radio-isotope labelling was increased. It is unlikely that the presence of HSPs in ES products was due to the damage of worms. During heat-shock treatment and isotope labelling, the worms were regularly checked under a stereomicroscope. They were found to be alive and active. Metabolic radio-isotope labelling also ensured that proteins detected in ES products were newly synthesized products rather than stored materials which leaked out due to damage. Also, if the presence of HSPs in ES was due to leakage, there should theoretically, be a decrease of HSPs in the somatic preparations. However, no such decrease was observed.

There is another report in the literature on the occurrence of HSPs in ES products of parasites. Estes & Teale (1991) isolated 2 proteins from the culture supernatant of a cestode, *Mesocestoides corti*. The proteins were homologous to HSP 70 and *Escherichia coli* GroEL families of stress proteins. The supernatant also stimulated an *in vitro* antibody response restricted to IgM and IgG_1. On the basis of lymphocyte proliferative assays and immunoblotting studies, the authors suggested that the stress proteins from ES products may play an integral part in the immune response to *M. corti* and the associated phenomenon of isotype restriciton.

ES HSPs of *Trichinella* may also play a role in host

immunity. The suggestion is based on the fact that ES HSP 45 and 53 of *T. spiralis* have similar molecular masses and isoelectric points as two of the well-known specific antigens isolated from the supernatant of worm culture in previous studies (Gamble *et al.* 1988; Ko & Yeung, 1989, 1991). Therefore, some of the well-known specific ES antigens of *T. spiralis* which are commonly employed in serodiagnosis may actually be stress proteins. But further studies are required to validate such assumption.

When *Trichinella* was given a heat-shock treatment at 43 °C, a marked increase in HSPs 70 and 86 was observed in the somatic extacts. Although HSP 70 is one of the most common conserved HSPs in eukaryotes, its synthesis in substantial quantity in *Trichinella* may have a distinct implication. HSP 70 has been incriminated as a 'molecular chaperone' which can escort and influence the conformation taken by proteins, thus facilitating their transport across membranes. This is supposedly carried out by hydrophobic interactions between HSP and its charge (Newport, Culpepper & Agabian, 1988; Pelham, 1988).

Leung (1995) demonstrated that the infective-stage larvae of *T. spiralis* secretes DNA-binding proteins. The latter bind only to mammalian but not piscine DNA. Therefore, in trichinellosis, HSP 70 may play a role during the early phase of tissue invasion by folding DNA-binding effector molecules to facilitate their transport (via other mechanisms) into the target myonuclei. However, further studies, especially on the molecular structure of the *Trichinella* HSP 70, are required to confirm this suggestion.

In some parasitic protozoans and trematodes e.g. *Leishmania*, *Trypanosoma*, *Plasmodium* and *Schistosoma*, HSP 70 has been reported as a conserved HSP (Newport *et al.* 1988; Polla, 1991; Maresca & Carratu, 1992). It has also been found as a major immunogen in *Brugia* spp., *Schistosoma mansoni* and *Onchocerca volvulus* (Selkirk *et al.* 1989; Hedstrom *et al.* 1988; Rothstein *et al.* 1989). However, the exact role of this HSP in parasitic adaptations has yet to be delineated.

Although HSP 86 is another major protein synthesized by *Trichinella* during heat shock, its role in the infectious process is also unknown. In *Schistosoma mansoni*, however, Johnson *et al.* (1989) reported that the reading frame of a 86 kDa antigen was highly homologous to those of large HSPs of *Sacchromyces cerevisiae* (HSP 90) and *Drosophila melanogaster* (HSP 83). Several HSPs of other parasites are also known to be immunogenic (Newport *et al.* 1988; Polla, 1991; Ortner *et al.* 1992). However, all the known major immunodominant and specific antigens of *T. spiralis* and *T. pseudospiralis* have substantially lower molecular masses (from 45 to 53 kDa) (Gamble & Graham, 1984; Ko & Yeung, 1989, 1991). Therefore, the importance of HSP 86 as an immunogen in trichinellosis awaits further studies.

This work was partly supported by a grant from the Hong Kong Research Council to R. C. Ko.

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