Journal of Helminthology (2004) **78,** 243–247 DOI: 10.1079/JOH2003225

Heat shock protein synthesis over time in infective *Trichinella spiralis* larvae raised in suboptimal culture conditions

J. Martinez[1]*, J. Perez-Serrano[1], W.E. Bernadina[2], I. Rincon[1] and F. Rodriguez-Caabeiro[1]

[1]Facultad de Farmacia, Departamento de Microbiologia y Parasitologia, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain: [2]Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, University of Utrecht, PO Box 80165, 3508 TD Utrecht, The Netherlands

Abstract

Changes in the viability, infectivity and heat shock protein (Hsp) levels are reported in *Trichinella spiralis* first stage larvae (L1) stored in 199 medium for up to seven days at 37°C. These conditions induce stress that the larvae, eventually, cannot overcome. After three days of storage, the infectivity and viability were unchanged, although higher Hsp70 levels were observed. After this time, larvae gradually lost viability and infectivity, coinciding with a decrease in Hsp70 and Hsp90 and an increase in actin (a housekeeping protein). In addition, a possibly inducible heat shock protein, Hsp90i, appeared as constitutive Hsp90 disappeared. No significant changes in Hsp60 levels were detected at any time. These results suggest that heat shock proteins initially try to maintain homeostasis, but on failing, may be involved in cell death.

Introduction

Trichinella spiralis is readily maintained in the laboratory and is therefore a good experimental model for studying parasite physiology. The expression of heat shock proteins (Hsps) under stress conditions has been studied in several parasite species (del Cacho *et al.*, 2001; Descoteaux *et al.*, 2002; Dobbin *et al.*, 2002) including some belonging to *Trichinella* (Ko & Fan, 1996; Vayssier *et al.*, 1999; Niak *et al.*, 2001). However, while a number of studies have investigated the role of Hsps in parasite viability and infectivity (Smejkal *et al.*, 1988; Miller *et al.*, 1999; Adhuna *et al.*, 2000; Descoteaux *et al.*, 2002), none have been performed with *T. spiralis*.

In the present study, infective larvae of *T. spiralis* (L1) were stored in 199 medium at 37°C for up to 7 days, conditions that eventually lead to their death. Such early death does not occur in other helminths which grow and differentiate *in vitro* (Smyth, 1990; Hemphill & Gottstein, 1995), probably due to differences in metabolism.

*Fax: 34 918854663
E-mail: francisco.martinez@uah.es

Heat shock proteins are good indicators of stress in *T. spiralis* L1 (Martinez *et al.*, 1999, 2002), and Hsp60, Hsp70 and Hsp90 levels were monitored by Western blotting until the larvae died. Viability (%), infectivity, and actin levels were also measured. The aim of the present study was to relate the possible role of Hsp levels to the viability and infectivity of *T. spiralis* larvae exposed to the above stress. This situation may be similar to that which occurs when larvae are rejected during reinfection.

Materials and methods

A single batch of first stage larvae (L1) of *T. spiralis* (isolate MFEL/SP/62/GM1/ISS 48; La Rosa *et al.*, 1992) was used for all procedures. Bacteria-free, viable (100%) L1 were obtained following the method of Martinez *et al.* (1999). Pooled larvae were counted three times using a standard protocol (Wood *et al.*, 1995). Aliquots (0.5 ml) containing approximately 7000 L1 were added to tubes with 6.5 ml pre-warmed (37°C) medium 199 (Sigma). These were then stored at 37°C for 0, 1, 3, 5 or 7 days (four tubes per condition). The medium was renewed every 24 h. Changes in infectivity were studied in parallel

https://doi.org/10.1079/JOH2003225 Published online by Cambridge University Press

mouse infection experiments. Four female (NMRI) mice were orally inoculated with 400 larvae raised under the different storage time conditions. These mice were sacrificed at day 35 post-infection and muscle larvae obtained by routine pepsin-HCl digestion. After counting, infectivity was expressed as the mean numbers of muscle larvae per gram of muscle.

Primary and peroxidase-conjugated mouse and rabbit secondary antibodies (Abs) (Sigma) were used for protein analyses. The primary Abs used were: monoclonal (m)Ab AC-16 to Hsp90, mAb BRM-22 to Hsp70, mAb LK2 to Hsp60, and actin (rabbit) Ab. In prior blotting experiments, mAb AC-16, BRM-22 and LK2 were found to produce linear response curves to Hsp90, Hsp70 and Hsp60 ($r > 0.95$, three replicates).

The Hsps and actin from *T. spiralis* L1 extracts were detected by Western blotting. Briefly, extracts were obtained by sonication of larvae at 4°C. Insoluble material was removed by centrifugation ($14000 \times g$, 20 min at 4°C) and the supernatants prepared for gel electrophoresis. Proteins (5 μg for Hsp90 and 15 μg for Hsp70 and Hsp60, in quadruplicate) were separated on 10% resolving gels (Laemmli, 1970) and transferred to Immobilon-P membranes (Millipore). After incubation in blocking buffer (150 mM phosphate-buffered saline, pH 7.2 plus 0.05%Tween 20 (PBS-T) plus 5% non-fat dry milk (Nestle), the blots were incubated with mAb AC-16 (1/10000) or mAb BRM-22 and LK2 (1/1000). Following incubation with 1/6000 diluted peroxidase-conjugated secondary Ab, positive bands were detected using the substrate 0.06% 3,3′diaminobenzidine plus 1/1000 diluted concentrated H_2O_2. Primary antibodies were incubated overnight at 4°C, while secondary antibodies were incubated for 2 h at room temperature. After each step, the membranes were washed three times with PBS-T. Protein bands were quantified using an image analyser and 1D image analysis software (Scion Image for Windows, Scion Corporation). Immunoreactivity (arbitrary units) was determined as the optical density × area of the band. Any statistically significant differences were evaluated by the Dunnet test.

Results

Figure 1a shows the immunoblot data for Hsp70 and Hsp60 at day 0 (controls) 1, 3, 5 and 7. Densitometric analysis of fig. 1a shows that Hsp70 levels were significantly higher by day 3 compared to day 0. However, Hsp70 levels dropped significantly by day 7 (fig. 1b), whereas no significant changes in Hsp60 levels were detected at any time (fig. 1c).

Hsp90 levels did not change as a result of storage for up to 3 days (fig. 2a,b). From day 5 onwards, however, they decreased and an inducible form of Hsp90 (Hsp90i) appeared (fig. 2a,c). The time course of the storage-induced reduction of Hsp90 paralleled that of Hsp90i increase.

The above studies required reliability controls. Three parallel control experiments were conducted to demonstrate overall good housekeeping function in each experimental L1 group. The Jenkins & Carrington (1981) protocol was used to identify dead *T. spiralis* L1

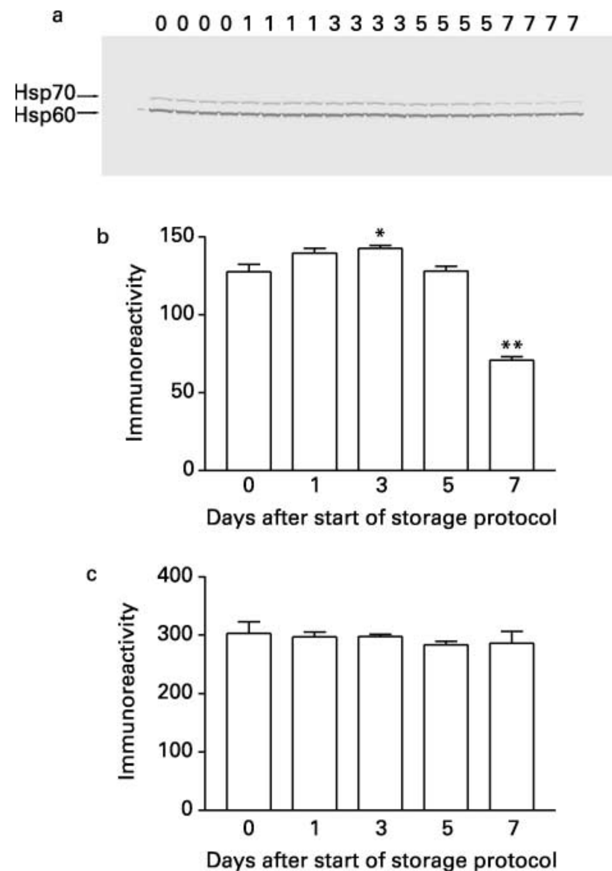

Fig. 1. Hsp70 and Hsp60 levels in *Trichinella spiralis* L1 after 0 (control), 1, 3, 5 and 7 days storage at 37°C in 199 medium. (a) Immunoblot data, (b) Hsp70 and (c) Hsp60 data obtained by densitometric analysis of fig. 1a (arbitrary units). Bars indicate mean ± SE of four replicates (*$P < 0.05$, **$P < 0.01$).

(i.e. non-motile and assuming a typical comma shape). Viability hardly altered after 5 days storage, but decreased to about 50% by day 7 (fig. 3a). On day 0 (controls), 1, 3, 5 and 7, larvae were used to infect mice. Muscle larvae burden analyses showed the infectivity of the L1 to be similar to that of controls (day 0) on days 1 and 3, but reduced by day 5 and lost by day 7 (fig. 3b). All experimental L1 groups contained housekeeping actin (stored or made for intracellular purposes in relation to normal and stress metabolism) in either normal or increased amounts.

Discussion

Trichinella spiralis larvae cannot moult in the culture conditions imposed and die within a few days. This situation may be similar to that occurring when larvae are rejected during reinfection. Rejected larvae can neither install themselves in the intestine nor moult. However, if larvae are transferred to a naive host, they can continue their life cycle (Wakelin & Denham, 1983).

Until their death, larvae are exposed to a state of stress, and they must activate cellular mechanisms to survive.

https://doi.org/10.1079/JOH2003225 Published online by Cambridge University Press

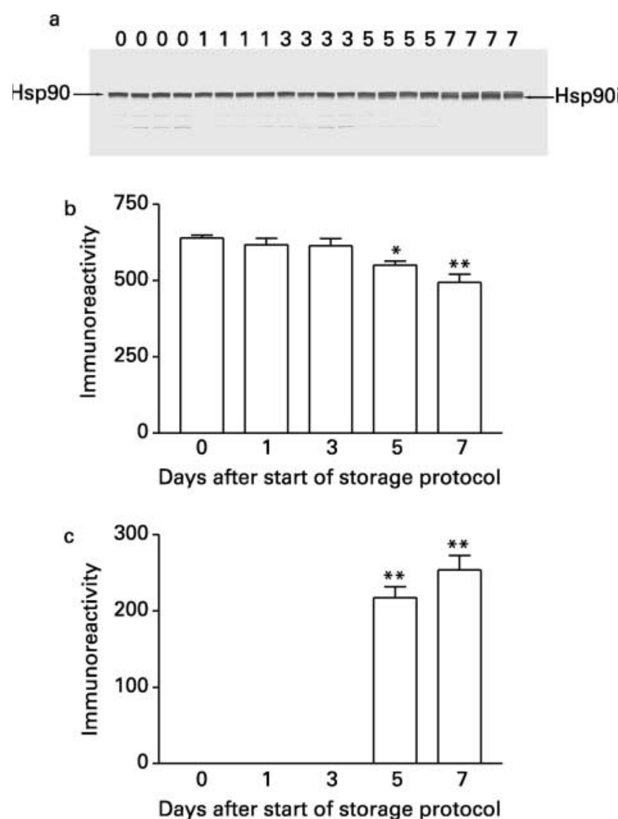

Fig. 2. Hsp90 levels in *Trichinella spiralis* L1 after 0 (control), 1, 3, 5 and 7 days storage at 37°C in 199 medium. (a) Immunoblot data, (b) constitutive and (c) inducible Hsp90 data obtained by densitometric analysis of fig. 2a (arbitrary units). Bars indicate mean ± SE of four replicates (*$P < 0.05$, **$P < 0.01$).

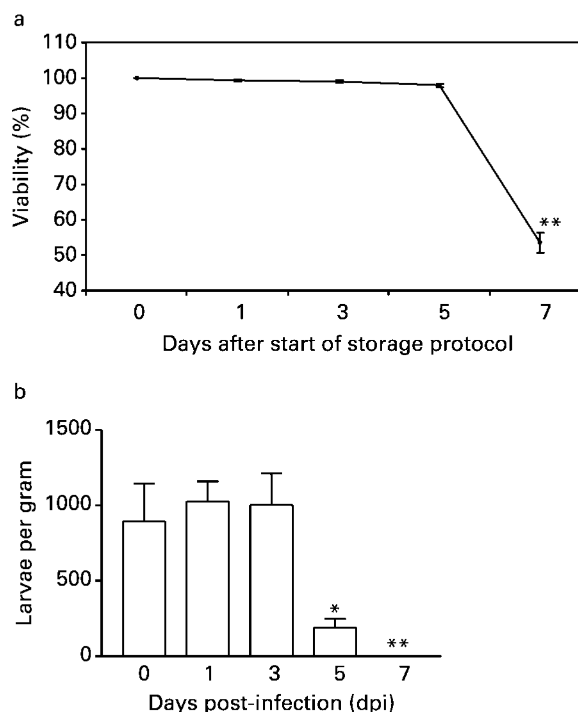

Fig. 3. (a) Viability of *Trichinella spiralis* L1 after 0 (control), 1, 3, 5 and 7 days storage at 37°C in 199 medium. Bars indicate mean ± SE of four replicates (**$P < 0.01$). (b) Infectivity of *T. spiralis* larvae after 0 (control), 1, 3, 5 and 7 days storage in the same conditions. Bars indicate mean numbers of muscle larvae per gram of muscle ± SE from four mice per experimental group (**$P < 0.01$).

It is well known that stress proteins prevent the loss of homeostasis due to their chaperone function (Hartman & Gething, 1996; Beissinger & Buchner, 1998), but they also have a role in protein degradation (Terlecky, 1994) and even in cell death (Wei *et al.*, 1995; Stamatis-Nick *et al.*, 1998; Vayssier & Polla, 1998). We hypothesize that the infectivity of larvae can only be maintained while stress proteins protect cellular homeostasis. Therefore, Hsp levels should increase in larvae exposed to stressful conditions before they lose their infectivity. When homeostasis is lost, this should coincide with a decrease in or lack of infectivity.

The present results show, for the first time, that *T. spiralis* L1 (ISS48) can retain full infective power for at least 3 days at 37°C in 199 medium. During this period, actin levels do not change significantly, although Hsp70 levels increase, indicating that storage between 1 and 3 days is stressful. Probably, Hsp70 levels increase to maintain homeostasis in *T. spiralis* L1 (corroborated by the unchanged actin level) and therefore its infectivity. Given the unchanged levels of both Hsp60 and Hsp90 during this time, it seems that neither have a major role in avoiding loss of infectivity. However, the fact that Hsp60 and Hsp90 levels remained constant may indicate that homeostasis was maintained since these proteins play a major role in cell mechanisms (Hartman & Gething, 1996; Pratt *et al.*, 1996).

On day 5, the infectivity, but not viability, of larvae decreased significantly. These events were accompanied by a non-significant decrease in Hsp70, a non-significant increase in actin, a significant decrease in Hsp90 (constitutive form), and the synthesis of an inducible form of Hsp90 (Hsp90i). It cannot be ruled out, however, that the latter is a degradation form of Hsp90. Taken together, these results may indicate that homeostasis was progressively lost between days 3 and 5, even though viability was around 100%. These observations may be of great importance in *T. spiralis* biology. It seems that a loss in infectivity in this parasite is a prelude to the loss of other vital functions. Indeed, the data indicate that a loss of infectivity precedes that of viability and normal actin levels. In addition, decreased Hsp90 levels have been related to apoptosis in other systems (Miyata, 2003; Vanden Berghe *et al.*, 2003). It is not known whether this occurs in this system, although proteolytic mechanisms would have been activated if Hsp90i were a degradation product. In recent years, proteolysis has emerged as a key process in apoptosis (Samali & Orrenius, 1998).

On day 7, L1 viability was halved but infectivity decreased from 20% to 0%. Levels of Hsp70 and constitutive Hsp90 decreased to below their levels on day 5, while Hsp90i and actin levels increased significantly. The levels of these proteins may be related

https://doi.org/10.1079/JOH2003225 Published online by Cambridge University Press

to the total loss of infectivity and much reduction in viability. Larvae die progressively, implying a loss of homeostasis and probably cell death, but it cannot be ruled out that factors or conditions beyond those addressed here contribute to the loss of infectivity observed. This lack of infectivity might be explained by a decrease in carbohydrate reserves in L1 isolated from mice carcasses 4 days after the animals were killed (Raines & Stewart, 1988), and/or by host factors. The latter are shown by the fact that in previous experiments, on day 9 of storage, *T. spiralis* L1 (viability 12% at this time) produces 10 ± 5.7 muscle larvae ($n = 4$) in CD1 mice (unpublished). This indicates that physiological and immunological factors are involved in the infectivity of larvae in agreement with that reported by Bell (1998). Thus, *T. spiralis* L1 should be regarded as living unless they acquire the typical comma-shape in accordance with the criterion of Jenkins & Carrington (1981).

In conclusion, fluctuations in Hsp levels may be related to the viability and infectivity of larvae since: (i) at the beginning, viability and infectivity is maintained despite the stressful conditions (homeostasis is probably maintained through the increase in Hsp70 levels); (ii) later infectivity is lost and some Hsps levels alter considerably (homeostasis may not be lost since viability does not decrease and actin levels do not change significantly, although falling infectivity and Hsp70 and Hsp90 levels suggest this is the case); (iii) the decrease in constitutive Hsp90 and Hsp70 levels may have a role in, or be an indicator of, programmed cell death (apoptosis or necrosis). Decreased levels of these proteins, both of which are essential for maintaining homeostasis, have been associated with cell death in other systems (Tosi *et al.*, 1997; Sapozhnikov *et al.*, 2002; Miyata, 2003; Vanden Berghe *et al.*, 2003). In fact, the induction of Hsp70 and/or Hsp90 has been associated with a suppression of apoptosis in many studies (Samali & Orrenius, 1998; Vayssier & Polla, 1998). Although it cannot be confirmed that apoptosis occurs in the present system, it is clear that a relationship exists between the reduction in Hsp90 and Hsp70 levels and the decreased viability and infectivity of larvae.

Acknowledgements

This study was funded by the Spanish Ministry of Science and Technology (reference AGL2000-1792).

References

Adhuna, A., Saltora, P. & Bhatnagar, R. (2000) Nitric oxide induced expression of stress proteins in virulent and avirulent promastigotes of *Leishmania donovani*. *Immunology Letters* **71**, 171–176.

Beissinger, R.P. & Buchner, J. (1998) How chaperones fold proteins. *Biological Chemistry* **379**, 245–259.

Bell, R.G. (1998) The generation and expression of immunity to *Trichinella spiralis* in laboratory rodents. *Advances in Parasitology* **41**, 149–217.

del Cacho, E., Gallego, M., Pereboom, D., Lopez-Bernad, F., Quilez, J. & Sanchez-Acedo, C. (2001) *Eimeria tenella*: hsp70 expression during sporogony. *Journal of Parasitology* **87**, 946–950.

Descoteaux, A., Avila, H.A., Zhang, K., Turco, S.J. & Beverley, S.M. (2002) *Leishmania* LPG3 encodes a GRP94 homolog required for phosphoglycan synthesis implicated in parasite virulence but not viability. *EMBO Journal* **21**, 4458–4469.

Dobbin, C.A., Smith, N.C. & Johnson, A.M. (2002) Heat shock protein 70 is a potential virulence factor in murine toxoplasma infection via immunomodulation of host NF-kappa B and nitric oxide. *Journal of Immunology* **169**, 958–965.

Hartman, D. & Gething, M.J. (1996) Normal protein folding machinery. pp. 3–24 *in* Feige, U., Morimoto, R.I., Yahara, I. & Polla, B.S. (*Eds*) *Stress-inducible cellular response*. Berlin, Birkhäuser Verlag.

Hemphill, A. & Gotstein, B. (1995) Immunology and morphology studies on the proliferation of *in vitro* cultivated *Echinococcus multilocularis* metacestodes. *Parasitology Research* **81**, 605–614.

Jenkins, D.C. & Carrington, T.S. (1981) An *in vitro* screening test for compounds active against the parental stages of *Trichinella spiralis*. *Tropenmedizin und Parasitologie* **32**, 31–34.

Ko, R.C. & Fan, L. (1996) Heat shock response of *Trichinella spiralis* and *T. pseudospiralis*. *Parasitology* **112**, 89–95.

La Rosa, G., Pozio, E., Rossi, P. & Murrell, K.D. (1992) Allozyme analysis of *Trichinella* isolates from various host species and geographical regions. *Journal of Parasitology* **78**, 641–646.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Martinez, J., Perez-Serrano, J., Bernadina, W.E. & Rodriguez-Caabeiro, F. (1999) *In vitro* stress response to elevated temperature, hydrogen peroxide and mebendazole in *Trichinella spiralis* muscle larvae. *International Journal for Parasitology* **29**, 1457–1464.

Martinez, J., Perez-Serrano, J., Bernadina, W.E. & Rodriguez-Caabeiro, F. (2002) Expression of Hsp90, Hsp70 and Hsp60 in *Trichinella* species exposed to oxidative shock. *Journal of Helminthology* **76**, 217–223.

Miller, C.M., Smith, N.C. & Johnson, A.M. (1999) Cytokines, nitric oxide, heat shock proteins and virulence in *Toxoplasma*. *Parasitology Today* **15**, 418–422.

Miyata, Y. (2003) Molecular chaperone HSP90 as a novel target for cancer chemotherapy. *Nippon Yakurigaku Zasshi* **121**, 33–42.

Niak, C.H., Su, K.W. & Ko, R.C. (2001) Identification of some heat-induced genes of *Trichinella spiralis*. *Parasitology* **123**, 293–300.

Pratt, W.B., Gehring, U. & Toft, D.O. (1996) Molecular chaperoning of steroid hormone receptors. pp. 79–96 *in* Feige, U., Morimoto, R.I., Yahara, I. & Polla, B.S. (*Eds*) *Stress-inducible cellular response*. Berlin, Birkhäuser Verlag.

Raines, K.M. & Stewart, G.L. (1988) Carbohydrate reserves and infectivity in *Trichinella spiralis* isolated from carrion. *Parasitology* **96**, 533–541.

https://doi.org/10.1079/JOH2003225 Published online by Cambridge University Press

Samali, A. & Orrenius, S. (1998) Heat shock proteins: regulators of stress response and apoptosis. *Cell Stress and Chaperones* **3**, 228–236.

Sapozhnikov, A.M., Gusarova, G.A., Ponomarev, E.D. & Telford, W.G. (2002) Translocation of cytoplasmic HSP70 onto the surface of EL-4 cells during apoptosis. *Cell Proliferation* **35**, 193–206.

Smejkal, R.M., Wolff, R. & Olenick, J.G. (1988) *Leishmania braziliensis panamensis*: increased infectivity resulting from heat shock. *Experimental Parasitology* **65**, 1–9.

Smyth, J.D. (1990) Nematodes: other than Filarioidea. pp. 187–229 *in* Smyth, J.D. (*Ed.*) *In vitro cultivation of parasitic helminths*. Boston, CRC Press.

Stamatis-Nick, C.L., Charles, C.V. & George, C.T. (1998) The alter ego of heat shock proteins. *Clinical Immunology and Immunopathology* **3**, 235–236.

Terlecky, S.R. (1994) Hsp70s and lysosomal proteolysis. *Experientia* **50**, 1021–1025.

Tosi, P., Visani, G., Ottaviani, E., Gibellini, D., Pellacani, A. & Tura, S. (1997) Reduction of heat-shock protein-70 after prolonged treatment with retinoids: biological and clinical implications. *American Journal of Hematology* **56**, 143–150.

Vanden Berghe, T., Kalai, M., Van Loo, G., Declercq, W. & Vandenabeele, P. (2003) Disruption of HSP90 function reverts tumor necrosis factor-induced necrosis to apoptosis. *Journal of Biological Chemistry* **278**, 5622–5629.

Vayssier, M. & Polla, B.S. (1998) Heat shock proteins chaperoning life and death. *Cell Stress and Chaperones* **3**, 221–227.

Vayssier, M., Le Guerhier, F., Fabien, J.F., Philippe, H., Vallet, C., Ortega-Pierres, G., Soule, C., Perret, C., Liu, M., Vega-Lopez, M. & Boireau, P. (1999) Cloning and analysis of a *Trichinella britovi* gene encoding a cytoplasmic heat shock protein of 72 kDa. *Parasitology* **119**, 81–93.

Wakelin, D. & Denham, D.A. (1983) The immune response. pp. 265–308 *in* Campbell, W.C. (*Ed.*) *Trichinella and trichinellosis*. New York, Plenum Press.

Wei, Y.Q., Zhao, X., Kariya, Y., Teshigawara, K. & Uchida, A. (1995) Inhibition of proliferation and induction of apoptosis by abrogation of heat shock protein (Hsp)70 expression in tumor cells. *Cancer Immunology and Immunotherapy* **40**, 73–78.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone, J.B., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M. & Vercruysse, J. (1995) World Association for the Advancement of Veterinary Parasitology (WAAVP) second edition of guidelines for evaluating the efficacy of anthelminthics in ruminants (bovine, ovine, caprine). *Veterinary Parasitology* **58**, 181–213.

(Accepted 23 October 2003)
© CAB International, 2004

https://doi.org/10.1079/JOH2003225 Published online by Cambridge University Press