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HEAT SHOCK PROTEINS HSP70 AND HSP60 IN *ECHINOCOCCUS GRANULOSUS* PROTOSCOLICES

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The heat shock response is a ubiquitous process described in prokaryotic as well as eukaryotic cells (Feige U., Mollenhauer J. 1992: Experientia 48: 621-622). This response is characterized by an induction, due to transcriptional activation, of a specific set of genes (Sorger P.K., Pelham H.R.B. 1987: EMBO J. 6: 993-998); the final products of this activation are the so called heat shock proteins (HSPs). HSPs play different functions in protein synthesis (as molecular chaperones) as well as in protein degradation (Burel C., Mezger V., Pinto M., Rallu M., Trigon S., Morange M. 1992: Experientia 48: 629-634). Furthermore, there are works where a hypothetical role for these proteins in immune response has been described (Kaufmann S.H.E. 1992: Experientia 48: 640-643; Mollenhauer J., Schulmeister A. 1992: Experientia: 48: 644-649), and it seems that they are also involved, directly or indirectly, in the development of autoimmune diseases (Cohen I.R. 1991: Rev. Immunol. 9: 567-589; Kaufmann S.H.E. 1991: Curr. Top. Microbiol. Immunol. 167: 1-214).

There are a lot of works about the HSP expression in mammals; however, the number of papers on parasite organisms is still low. Whatever the case, it has been previously demonstrated that this protein expression could play a fundamental role in the virulence of parasites (Smejkal R.M., Wolff R., Olenick J.G. 1988: Exp. Parasitol. 65: 1-9), their differentiation (Van der Ploeg L.H.T., Giannini S.H., Cantor C.R. 1985: Science 228: 1443-1446) and in the host adaptation (Maresca B., Carratù L. 1992: Parasitol. Today 8: 260-266).

The pattern of these protein expressions has been studied in different parasites such as *Trypanosoma*, *Leishmania*, *Trichomonas, Trichinella* and *Haemonchus* (Alcina A., Urzainqui A., Carrasco L. 1988: Eur. J. Biochem. 172: 121-127; Mc Farlane J., Blaxter M.L., Bishop R.P., Miles M.A., Kelly J.M. 1990: Eur. J. Biochem. 190: 377-384; Davis S.R., Lushbaugh W.B. 1992: Am. J. Trop. Med. Hyg. 47: 70-77; Van Leewen M.A.W. 1995: Parasitol. Res. 81: 706-709; Ko R.C., Fan L. 1996: Parasitology 112: 89-95); however, the papers where the presence of HSPs in the Excretion-Secretion (ES) products has been described are fewer, as far as we know only in *Trichinella* (Ko and Fan 1996, op. cit.) and *Mesocestoides* (Estes D.M., Teale J.M. 1991: J. Immunol. 147: 3926-3934).

In this report we study the HSP90, HSP70, HSP60 and HSP25 expression in *Echinococcus granulosus* protoscolices in control conditions and after heat shock treatment, both in crude somatic extract of protoscolices and in ES products.

E. granulosus protoscolices were aseptically collected from liver hydatid cysts in sheep slaughtered at the municipal abattoir in Alcalá de Henares, Spain. Viability prior to testing was 95-99% as assessed by the methylene blue exclusion test and microscopic examinations, as previously described (Casado N., Rodríguez-Caabeiro F., Hernandez S. 1986: Z. Parasitenkd. 72: 273-278). The organisms (25,000 protoscolices/Leighton tube) were cultured in 3 ml of medium 199 (Sigma). *In vitro* incubations were performed at different temperatures, these being: 37°C, 42°C and 45°C for 4 h. At the end of the incubations, the medium was removed, centrifuged (10 min; 12,000 *g*) and precipitated with 10% TCA (trichloroacetic acid). This solution was centrifuged at 50,000 *g* for 20 min at 4°C. The pellet was resuspended in phosphate-buffered saline (PBS) and stored at -70°C. Protoscolices were homogenized in 0.5 ml of PBS by sonicator. This homogenate was centrifuged at 100,000 *g* for 30 min at 4°C. The supernatant was collected and the protein concentration was determined (Bradford, Bio-Rad). Samples (20 μg/well) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Stacking gels containing 4% and separating gels 12% acrylamide were routinely used. Electrophoresis was carried out at a constant current of 40 mA. Electroblot transfer from the polyacrylamide gels was performed as described by Towbin P., Staehelin T., Gordon J. 1979: Proc. Nat. Acad. Sci. USA 76: 4350-4354). The nitrocellulose blots were washed in PBS containing 0.05% Tween-20, and incubated with 5% non fat powdered milk for 1 h. After the incubations, blots were tested with antisera. Primary monoclonal antibodies (Sigma) were: anti-HSP90 (1/500 dilution), anti-HSP70 (1/5,000 dilution), anti-HSP60 (1/1,000 dilution) and anti-HSP25 (1/500 dilution), diluted in PBS. The peroxidase conjugated secondary antibody (Sigma) was used at 1/1,000 dilution. The peroxidase reaction was carried out of 0.05M Tris buffer containing 0.03% diaminobenzidine (DAB), and 0.001% hydrogen peroxide. For densitometric quantification of blot immunoreactivity a digital analysis image system (Microm, Spain) was used.

Immunoblotting revealed that HSP60 and HSP70 were present in the ES products of 42°C and 45°C heated proto-

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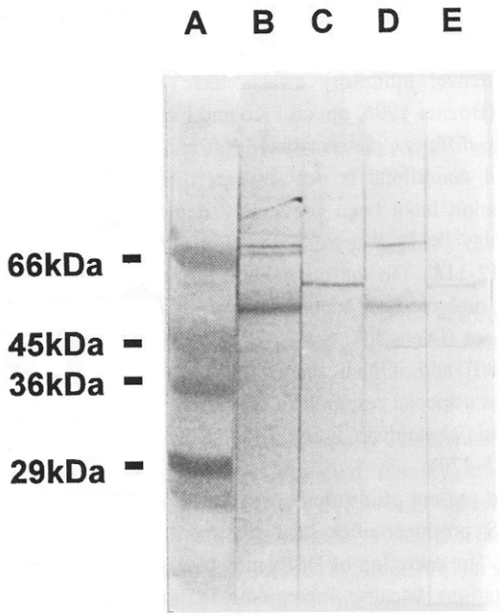

Fig. 1. Immunoblotting of ES products of protoscolices of *Echinococcus granulosus*. **A –** Molecular weight markers; **B** – HSP70 of 42°C heated protoscolices; **C** – HSP60 of 42°C heated protoscolices; **D** – HSP70 of 45°C heated protoscolices; **E –** HSP60 of 45°C heated protoscolices.

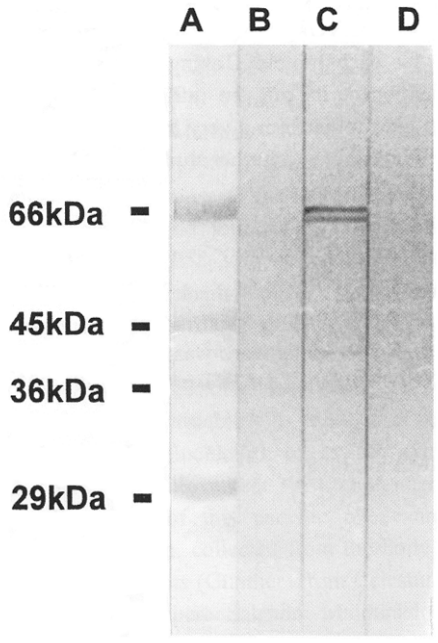

Fig. 2. Immunoblotting of protoscolices somatic crude extract incubated with anti-HSP70. **A –** Molecular weight markers; **B –** 37°C heated protoscolices; **C –** 42°C heated protoscolices; **D –** 45°C heated protoscolices.

scolices treated (Fig. 1), but not at 37°C. The presence of these HSPs in the medium as ES products of *in vitro* cultured

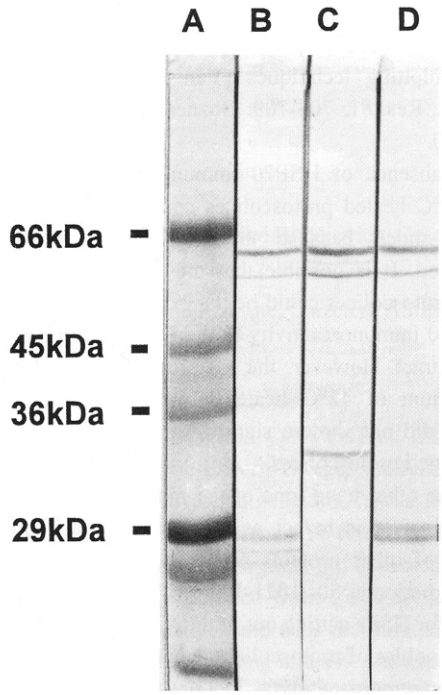

Fig. 3. Immunoblotting of protoscolices somatic crude extract incubated with anti-HSP60. **A –** Molecular weight markers; **B** – 37°C heated protoscolices; **C** – 42°C heated protoscolices; **D –** 45°C heated protoscolices.

parasites has also been described before (Estes and Teale1991, op. cit.; Ko and Fan 1996, op. cit.). They could play a very important role in the host-parasite relationship, mainly in the immune response, since antibodies against HSP of parasite origin have been found in host sera (Nagasawa H., Oka M., Maeda K-I., Jian-guo C., Hisaeda H., Ito Y., Good R.A., Himeno K. 1992: Proc. Nat. Acad. Sci. USA 89: 3155-3158; Quijada L., Requena J.M., Soto M., Alonso C. 1996: Parasitology 112: 277-284). Neither HSP90 nor HSP25 were found either in ES products or crude somatic extracts. It must be considered that the anti-HSPs antibodies were raised against mammalian HSP. It could be possible that these antibodies only recognize the HSP60 and HSP70, the most conserved HSPs in the evolutive scale (Wynn R.M., Davie J.R., Cox R.P., Chuang D.T. 1994: J. Lab. Clin. Med. 124: 31-36).

As can be seen in the ES products (Fig. 1) we found another protein of a molecular weight of 50 kDa (approx.), which could be another HSP, an unrelated protein that shares an epitope similar to the HSP70 epitope or a specific cleavage of HSP70 protein (Mitchell H.K., Petersen N.S., Buzin C.H. 1985: Proc. Nat. Acad. Sci. USA. 82: 4969-4973).

HSP70 was only seen in crude somatic extracts of 42°C treated protoscolices, not being present at other incubation temperatures (Fig. 2), although this protein was found in ES products from 42°C and 45°C heated protoscolices. HSP70 has also been observed and induced in other parasites. This

event was demonstrated using autoradiographic techniques (Alcina et al. 1988, op. cit.; Ko and Fan 1996, op. cit.) and immunoblotting techniques (Van Leeuwen M.A.W. 1995: Parasitol. Res. 81: 706-709; Bozner P. 1996: J. Parasitol. 82: 103-111).

The absence of HSP70 immunoreactivity in the extract from 45°C heated protoscolices could be due to this strong thermic shock because their viability was significantly diminished. It is possible that the death and lysis of 45°C heated protoscolices could be the explanation for the presence of HSP70 immunoreactivity in the ES products but not in the crude extract. However, the ES products were also found in the medium of 42°C heated protoscolices althought their viability did not show a significant reduction with regard to 37°C heated protoscolices.

On the other hand, one of the most accepted roles for the HSP70 protein is to act as a chaperone, facilitating translocation of other proteins across membrane (Terlecky S.R. 1994: Experientia 50: 1021-1025). This mechanism could be the way for HSPs getting out of the 42°C heated protoscolices.

Immunoblot of protoscolices somatic crude extract showed HSP60 immunoreactivity at 37°C, which increased in blots of 42°C and 45°C heated protoscolices (Fig. 3). HSP60 immunoreactivity was quantified with a digital image analysis system and was 43% higher at 42°C than at 37°C; and 28% higher at 45°C than at 37°C. HSP60 has also been pointed out before with the same monoclonal antibodies (clone LK2) in another parasite as *Haemonchus* (Van Leeuwen 1995, op. cit.), but it did not show an increase in the amount of HSP60 in the different immunoblots after heat shock treatments, such as we have seen. However, it had been previously reported that HSP60 is a mitochondrial protein that is constitutively expressed and heat-induced (Mc Mullin T.W., Hallberg R.L. 1987: Mol. Cell. Biol. 7: 4414-4423).

Heat stress is, if not the best, one of the better systems to induce HSP expression. However, in the present study, we demonstrate HSP60 and HSP70 induction only after 42°C hyperthermic treatment; no significant inductions were demonstrated after 45°C treatment. It is possible that this level of heat stress could modify protein synthesis. It has been demonstrated that protein synthesis shows a temperature dependence; inhibitory effects have been described above 43°C (Bozner 1996, op. cit.; Ko and Fan 1996, op. cit.).

The different pattern of HSP60 and HSP70 expression in control conditions is not strange, different levels of HSPs expression have been previously demonstrated in mammals (Tanguay R.M., Wu Y., Khandjian E.W. 1993: Dev. Genet. 14: 112-118). On the other hand, different patterns of HSPs induction have been reported in vertebrates after hyperthermic treatment (Dietz T.J., Somero G.N. 1993: Physiol. Zool. 66: 863-880) and it also seems clear that each type of stress induces a special response in relation to the induction of HSPs synthesis (Latchman D.S. 1995: Neuropathol. Appl. Neurol. 21: 475-477).

The present study demonstrates the presence of HSPs in the ES products after heat treatment of protoscolices "in vitro". The secretion of HSPs may be also produced in natural parasitations because increments in host temperature could induce secretion of HSPs. The strong antigenicity of these proteins, used as adjuvant by other authors (Del Guidice G. 1994: Experientia 50: 1061-1066), can support the hypothesis that HSPs could act as antigens of recognition elusion for immune response.

As far as we know, this is the first paper where the HSP70 and HSP60 expression of *E. granulosus* protoscolices has been described, demonstrating its presence in the culture medium as an ES product and with different pattern of induction for each protein. Taking into account all of the above mentioned it can be affirmed that this protein expression goes to perform a very important role in the host-parasite relationship, and further studies must be carried out in this way.

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