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Short Note

Heat Shock Protein Expressed by the Adult Fluke, *Clonorchis sinensis* in Vitro

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Jyh-Wei Shin, Ching-Yu Chen and Shiu-Nan Chen (1995) Heat shock protein expressed by the adult fluke, *Clonorchis sinensis* in vitro. *Zoological Studies* **34**(4): 281-283. Mature flukes of *Clonorchis sinensis* were recovered from the gall bladder and bile ducts of infected cats. Flukes were washed with sterilized normal saline, passed through a serial sterile procedure and incubated at 37°C in a 5% CO_2 atmosphere. After one week, flukes were changed to fresh culture medium and prepared for heat shock treatment. Treatment conditions are described as follows: 4°C for 120 min, followed by 25, 37 or 42°C each for 60 min, and 45°C for 30 min. SDS-PAGE of *C. sinensis* adult fluke extracts revealed a complex pattern of 34 polypeptide components which ranged from 8.5 to 112 kDa. When temperature rose to 42°C, two significant *C. sinensis* heat shock proteins (75 and 82 kDa) appeared. Results of electrotransfer and immunoblotting showed that the sites of heat-induced antigens can be recognized when using a sera pool of 25 clonorchiasis patients.

Key words: Chinese liver fluke, Stress protein.

Heat shock proteins (HSPs) were initially recognized by their increased synthesis after exposure of cells to elevated temperatures (Ritossa 1962, Lindquist 1986). Subsequently, a number of exciting findings stimulated interest in HSPs. It is now realized that HSPs play a role in the immune response to many bacterial and parasitic pathogens. Several trematode pathogens, such as *Schistosoma mansoni*, exposed to heat stress conditions, may form HSPs on the surface of schistosomulae (Taylor et al. 1984). Furthermore, HSPs may act as antigens and stimulate the humoral immune response (Shinnick 1991). Mature flukes, *Clonorchis sinensis*, live in the intrahepatic ducts, bile ducts, and gall bladders of many mammalian hosts. Some clinical clonorchiasis patients have fever symptoms at the beginning of their infection. Because both *S. mansoni* and *C. sinensis* are trematodes, we can speculate that *C. sinensis* can form the HSPs after heat shock treatment and that HSPs can stimulate a humoral immune response in the host. In this study, we describe a culture method for raising flukes and determine whether HSP expression by mature flukes of *C. sinensis* can be evoked in vitro.

Materials and Methods—Metacercariae of *C. sinensis* were harvested from the fresh water fish *Hemiculter kneri*, collected from Sun Moon Lake, Nantou County, Taiwan. Five male domestic cats (*Felis domestica*), which were two years old, were each infected orally with 100 *C. sinensis* metacercaria, respectively. After three months, mature flukes were recovered from the gall bladders and bile ducts of the infected cats. Flukes were washed with sterilized normal saline (SNS) until visually clean. Worms were carefully transferred to washing solution (SNS containing 1,000 U/ml penicillin-G, 50 μg/ml streptomycin sulfate and 10 μg/ml fungizone; GIBCO Laboratories, USA) by using tweezers and were incubated at 37°C for 30 min. After drawing off the supernatant, worms were transferred into soaking solution (minimum essential medium, MEM; FLOW Laboratories, USA, to which was added 100 U/ml penicillin-G and 5 μg/ml streptomycin sulfate) and incubated at 37°C for 30 min. Next 25 undamaged adult flukes were removed by using tweezers, whose tips were covered by plastic sheaths, placed into a 50 ml sterilized beaker with 20 ml culture medium (MEM plus 10% fetal calf serum; GIBCO Laboratories, USA) and incubated at 37°C for 30 min. Flukes were then transferred into a 25 cm^2 culture flask (Nunc, Denmark) to which 10 ml fresh culture medium was added and incubated at 37°C in a 5% CO_2 atmosphere. After one week, flukes were changed to fresh culture medium and prepared for heat shock treatment. Adult flukes were washed three times by using soaking solution, then 10 ml MEM was added to the flask and the flask was sealed tightly with paraffin film. The culture flasks, with 25 undamaged worms per flask, were treated at several different temperatures and time periods by using a separatory water bath. Treatment conditions are

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described as follows: 4°C for 120 min, 25°, 37° or 42°C each for 60 min, and 45°C for 30 min. Worms were separately collected from the culture flask. They were washed three times with extraction buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Triton X-100, 5 mM EDTA and 2 mM PMSF as modified by Bowtell et al. (1983)). The soluble protein was extracted from the worms with extraction buffer by using a homogenizer (Polytron PTA 20S homogenizer; Kintmatica, Switzerland), five times at 10,000 rpm for one minute intervals in an ice-water bath. The homogenate extract was centrifuged at 7,200 g for 30 min; then the supernatant was collected and stored separately at −20°C for the experiments. Protein concentrations on all samples were measured by use of Bio-Rad Protein Assay reagent from Bio-Rad Laboratories, USA. Next, 10~15% sodium dodecyl sulfate gradient polyacrylamide gel slabs (SDS-PAGE) were used for electrophoresis and five different heat shock samples were applied per well (25 μg/well). Polypeptide bands patterns were revealed by using the silver nitrate staining method as modified by Merril et al. (1979) Protein transfer and blotting techniques of Gershoni and Palade (1983) were modified slightly.

Results—SDS-PAGE of *C. sinensis* adult flukes exposed to different temperatures in the presence of 4% SDS and 50 mM dithiothreitol revealed a complex pattern with 34 polypeptide components which ranged from 8.5 to 220 kDa based on use of a Pharmacia calibration kit. (Pharmacia, USA.) Under 4°, 25° and 37°C treatments, polypeptide patterns (from 60 to 220 kDa) did not exhibit obvious differences changes. (Fig. 1A, lane 1-3) When the incubation temperature was 42°C, two significant *C. sinensis* heat shock proteins (CSHSP-75 and CSHSP-82) appeared (Fig. 1A, lane 4) at the sites of 75 and 82 kDa. At the higher temperature, 45°C, these polypeptide pattern was again like that of the 37°C treatment (Fig. 1A, lane 5). Results of electrotransfer and immunoblotting showed that CSHSPs sites could be recognized when using a sera pool of 25 clonorchiasis patients (Fig. 1B), but could not be recognized when using a sera pool of 25 normal persons sera pool (data not shown).

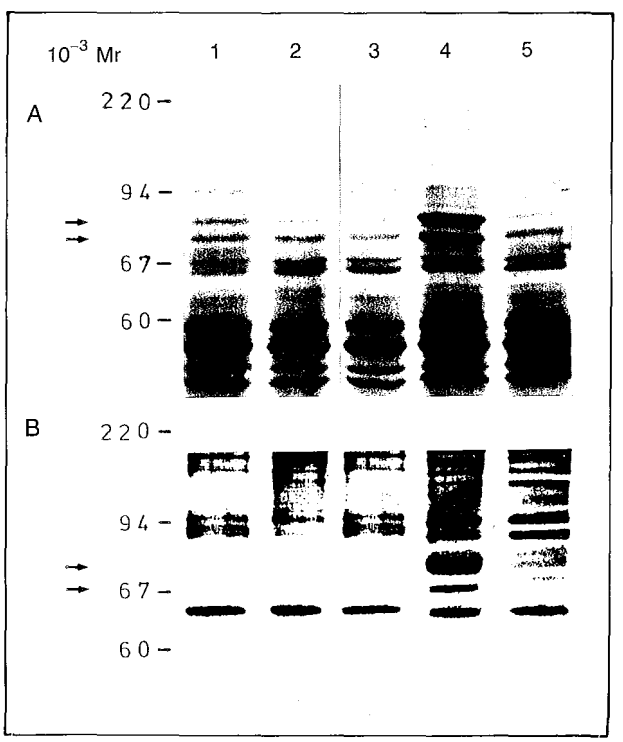

Fig. 1. A. Results showing polypeptides separated by SDS-PAGE from extracts of adult flukes *Clonorchis sinensis* after heat shock treatment. B. Immunoblotting of adult fluke *C. sinensis* extracts after heat shock treatment by using 25 clonorchiasis patients sera pool. Lane 1: 4°C for 120 minutes. Lane 2~4: 25°, 37°, and 42°C each for 60 minutes. Lane 5: 45°C for 30 minutes. The two arrows indicate the position of the heat induced antigens.

Discussion—It has been proposed that the heat shock response plays a fundamental role in parasites during host invasion (Newport et al. 1988). HSPs have been detected in many parasites (such as *Plasmodium, Leishmania, Trypanosoma, Schistosoma, Onchocerca*, and *Brugia* (Shinnick 1991)) at the time of infection. Dimorphic parasites, such as *S. mansoni* and *C. sinensis*, which shift between poikilothermic vectors at 22°-28°C and in another host, must adjust to the constant temperature of a homeothermic mammalian host at 37°C. It is possible that during host invasion the stress response by the parasite is elicited independently of the response to temperature (Maresca and Carratu 1992). When the metacercariae of *C. sinensis* shift from a fresh water fish to a human by accidental ingestion, the environmental temperature is changed very quickly, possibly causing the worm to produce HSPs under this circumstance. In this study, when the environmental temperature was 42°C, CSHSP-75 and CSHSP-82 were found in *C. sinensis* in vitro. In our study, the CSHSPs which were induced at 42°C from adult flukes of *C. sinensis*, strongly resemble the SDS-PAGE sites from the 25 clonorchiasis patients sera pool. That was not the case in other temperature treatments. Virtually all sera from the humans and animals infected with *S. mansoni* show apparent presence of an HSP. In mice, antibodies to this protein arise shortly after infection and are correlated with immune protection (Hedstrom et al. 1987). Those results were similar to the HSPs expressed from *C. sinensis* in vitro.

After excystation, metacercariae of *C. sinensis* rapidly attack the mucosa, and form a burrow in the duodenum. Responding to chemotaxis, larvae migrate along the common bile duct and locate at the bile canaliculi. At the moment of host invasion, an intense temperature adjustment occurs. HSPs derived from the larvae may function in thermotolerance (Maresca and Carratu 1992) and stage differentiation may trigger proteins (Lathigra et al. 1991). And it is possible that CSHSPs have a protective effect during the initial stages of transfer to a warm-blooded host (Maresca and Carratu 1992). It is clear that HSPs are immunologically identifiable components of many parasites. For several parasites, the humoral immune response to the HSP is directed predominantly towards nonconserved epitopes (Newport et al. 1988), and may be a part of parasite survival strategy. CSHSPs were not the only antigens detected during the in vitro studies, but since there was a specific immune response to these proteins, it is possible that these CSHSPs may be useful in immunodiagnosis of clonorchiasis.

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中華肝吸蟲熱休克性蛋白質之表現

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將人工感染的家貓體內所取得之中華肝吸蟲成蟲經系列無菌處理並馴養一週後，分別以4、25、37、42與45℃的不同溫度處理蟲體。利用SDS-PAGE分離蟲體蛋白質組成後發現，蟲體在經過42℃處理30分鐘後，在75與82 kDa處分別有蛋白質增加之現象。經中華肝吸蟲症患者血清混合液與蟲體研磨液進行免疫轉印反應後也有相類似之結果。由此可知中華肝吸蟲成蟲在體外培養下，經熱處理後會有75與82 kDa的熱休克性蛋白質的表現，同時該熱休克性蛋白質亦具有抗原性。

關鍵詞：中華肝吸蟲，緊迫蛋白質。

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