นิพนธ์ต้นฉบับ

Studies on production of excretory-secretory products from 3 rd stage larvae of gnathostoma spinigerum by an in vitro cultivation.

Wilai Rattanasiriwilai* Kasin Chanthachume*
Medhi Kulkumthorn* Paisarl Yingyourd*

Rattanasiriwilai W, Chanthachume K, Kulkumthorn M, Yingyourd P, Studies on production of excretory-secretory products from 3 rd stage larvae of gnathostoma spinigerum by an in vitro cultivation. Chula Med J 1985 Oct; 29 (10) : 1115-1120

Laboratory cycling of G. spinigerum was set up using cats, cyclops and mice as the definitive, first intermediate and second intermediate hosts respectively. Third stage larvae (L_3) were maintained metabolically active in vitro for an extended period in Earle's basal medium (EME); they appeared most active during the first 4-6 days in culture. The survival period could be enhanced when BME was supplemented with human serum although similar effects could be achieved with albumin. Another sera or macromolecular components (S) have been suggested to maintain the larvae in vitro. The excretory-secretory (ES) products released metabolically from the worms in BME were detected and showed high activity at day 4. These culturing fluids also reacted with anisera in enzyme-immunoassay, and such in vitro cultivation system may be utilised to produce large quantities of ES products which may express the potential or functional antigen for Gnathostomiasis.

วิไล รัตนศิริวิไล, เกศิน จันทชุม, เมธี กุลกำม์ธร, ไพศาล ยิ่งยวด, การศึกษาการผลิตสิ่งขับปล่อยสิ่งคัดหลั่งของตัวอ่อนพยาธิตัวจี๊ดระยะที่ 3 จากการเลี้ยงในหลอดทดลอง. จุฬาลงกรณ์เวชสาร 2528 ตุลาคม; 29(10) : 1115-1120

ตัวอ่อนระยะที่ 3 (L_3) ของพยาธิตัวจี๊ด ได้ทำการเก็บเกี่ยวจากการจัดตั้งวงชีพของพยาธิตัวจี๊ดขึ้นในห้องปฏิบัติการโดยใช้แมว, กุ้งไร และหนูถีบจักร เป็นโฮสท์ของพยาธิตัวแก่, โฮสท์ตัวกลางที่ 1 และ 2 ตามลำดับ นำพยาธิ L_3 นี้มาเลี้ยงในหลอดทดลอง โดยใช้ Earle's basal medium (BME) เป็นอาหารเลี้ยงเชื้อซึ่งทำให้ L_3 ดำรงชีวิตเคลื่อนไหวได้ว่องไวเป็นเวลานานพอควร โดยมีความว่องไวในการเคลื่อนไหวมากในช่วงวันที่ 4-6 การดำรงชีพของพยาธิ L_3 จะอยู่ในหลอดทดลองได้นานมากขึ้นเมื่อเติมเซรุ่มคนปกติหรือเติมอาบูมินที่เป็นโปรตีนสกัดจากเซรุ่มลงใน BME การจัดเลี้ยงพยาธิ L_3 ในหลอดทดลองนั้น ทำให้มีสิ่งขับปล่อย-สิ่งคัดหลั่ง (ES) จากขบวนการเมตาบอลิซึ่มของพยาธิซึ่งตรวจพบได้ และพบว่ามีปริมาณสูงสุดในช่วงวันที่ 4 ซึ่ง ES นี้พบมีปฏิกริยาทางภูมิคุ้มกันกับเซรุ่มคนไข้โรคพยาธิตัวจี๊ด จากการทดสอบด้วยวิธีเอ็นไซม์ (enzyme-immuno assay, ELISA) นับได้ว่าวิธีการทดลองเลี้ยง L_3 ได้ในหลอดทดลองนี้ น่าจะเป็นหนทางนำมาสู่การได้ ES ของพยาธิซึ่งอาจจะแสดงความเป็นแอนติเจน ซึ่งมีศักยภาพ (potential) หรือ ปฏิบัติภาพ (Functional) ได้ดีในโรคพยาธิตัวจี๊ด

* Department of Parasitology, Faculty of Medicine, Chulalongkorn University.

Gnathostomiasis caused by Gnathostoma spinigerum is one of the disease frequently reported in Southeast Asian countries including Phillippines, Indonesia, Vietnam, Cambodia, Loas, Burma, Malaysia and Thailand. It has been estimated that the highest incidence of human gnathostomiasis is found in Thailand.[1] Although the disease is of medical concern and a public health problem, little is currently known about the biology of the parasite, the specific diagnosis and treatment of the disease except the parasitic life cycle.[2] A considerable limitation in the investigative process is the problem of maintaining the parasitic cycle in the laboratory.

The classical approach to the preparation of many parasitic diagnostic antigens using crude worm extracts including those of Gnathostoma has been causing problems of underestimation.[3,4] Such antigens exhibit high contents of non-reactive or cross reactive components. Many extensive investigations have therefore been focused at present on the soluble exoantigen which is biologically less complex. These metabolic products which are actively released from the worm have high potential antigens and are more involved in the disease process.[5]

Future investigations on the antigenicity of metabolic products, should be in the direction of obtaining these. We demonstrated a laboratory method of maintain the Gnathostoma life cycle and the in vitro conditions which would lead to the availability of the excretory-secretory (ES) products of third stage G. spinigerum larvae (L_3).

Meterial and Methods

Laboratory cycling of Gnathostoma spinigerum

G. spinigerum was maintained in the laboratory by cycling in cats, cyclops and mice. In brief, the cats used as definitive hosts were infected by skin penetration with advanced third stage larvae* described by Daengsvang et al[6]. Approximately 8 months after infection, fertilized eggs were found in the stool and were concentrated by floatation in a saturated sodium chloride solution. The excess sodium chloride in the eggs suspension was washed out.

First stage larvae were hatched during the first 5-10 days by placing the eggs in a petri-dish partially submerged in distilled water and maintained at room temperature. The cyclops used as first intermediate hosts were the four species described by Sooksri[7]. They were obtained from a natural pond in Bangkok. The first stage larvae would be ingested by the cyclops after being kept together in a beaker containing distilled water and placed at room temperature. During the first 1-10 days of infection, the larvae in the body cavity of the cyclops would develope into early third stage larvae.

The procedure for infecting the mice with the fully developed larvae in cyclops was by using a gastric tube and force feeding. Each 3 months old mouse was fed with approximately 10 infected cyclops, with about 30-40 developed larvae. Under these conditions, the larvae migrated to the liver and were found to be advanced third stage mostly on day 15-30; later the infection was established in muscle and

*(These larvae were originally obtained from the Faculty of Tropical Medicine of Mahidol University.)

various organs. Some mice died during the 15-20 days and the larvae harvested from their livers were fed to other normal mice. After months of infection, all of the surviving mice were used as stock of advanced third stage larvae.

Method of collecting third-stage larvae

The infected mice were killed by ether anesthesis. The larvae were dissected from cysts in various organs by compressing the tissue between two glass plates under the dissecting microscope. All larvae were decontaminated by soaking and washing 3 times in Hank's balanced salt solution (HBSS) containing 800 I.U.penicillin, 800 ug streptomycin and 2 ug fungizone per ml. The larvae were left in contact with the antibiotics for a total of 60 mins.

In vitro cultivation method

The intact and actively motile larvae were selected and transferred to sterile disposable plastic multidish culture plates (Nuclon 146485, Nunc, Kamstrup, Denmark.) with sterile lids, containing Earle's basal medium (BME, Earle, 1966; GIBGO, Grand Island, New York, U.S.A.). Medium has been stated to consit of Earle's salts, vitamin, glucose, amino acids and L-glutamine, without sodium bicarbonate and phenol red.

In the experiment, 10-12 larvae were suspended in 1.5 ml. of medium per well. All media contained penicillin and streptomycin at final concentrations of 200 I.U./ml and 200 ug/ml, respectively. Incubation was carried out at 37° C in a humidified incubator with 5% CO_2 replaced by fresh culture medium every 48 hrs. The larvae were observed daily under the dissecting microscope and those which had lost mobility and movement of the body and head-bulb were removed to minimize the possibility of contaminating the ES products with materials released from dying worms. These products in the culture - media were monitored by their changing optical density at 280 nm.

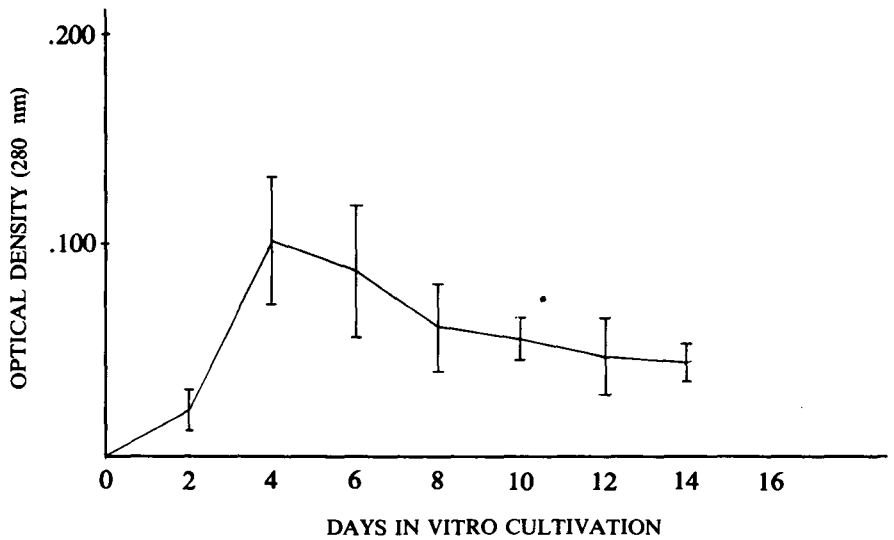

FIGURE The kinetic of metabolic activity of L_3 G. spinigerum, 10-12 worms were culture in 1.5 ml of BME and incubate at 37 C 5% CO_2 atmosphere. The medium was replaced at interval and its optical density at 280 nm was determined, using uncultured BME as blank.

Collection and antigen detection of ES products.

The fluid containing the larvae metabolic products were pooled, centrifuged at 2,000 rpm for 20 min to remove insoluble debris, and concentrated by ultrafiltration using a Diaflow membrane PM-10 (Amicon, Lexington, Mass, U.S.A.). These concentrated products were freed from the medium and reconcentrated after adding a large volume of normal saline. The protein contents of ES products were determined by the Folin-Ciocalteau method (Lowry et al., 1951).

The antigen in ES products was detected by an enzyme immuno-assay[9] (Voller et al. 1976), using the sera of patients with migratory swelling of cutaneous gnathostomiasis or from whom worms had been removed.

Results

ES production in BME media

The kinetics of ES production in BME media is shown in Figure. The optical density of the medium reached a peak during the first few days of incubation. It declined thereafter to a constant low activity for a period of time. The optical density was undetectable in the late phase of cultivation, when the larvae were not as active as in the early phase. However, the worms still survived because of the movement of lips. The high metabolic activity and the majority of proteins released during the early phase seemed to coincide with their environmental-adaptative activity. In the concentrated ES products, antigen could be detected by an enzyme-immuno assay with sera from Gnathostoma infected patients.

Survival of L_3 in different culture media

The results (Table) showed that the survival times of larvae in BME medium were similar to those in HBSS but the optical density at the preliminary determination by using their uncultured-matched medium as blank, was higher in BME. In addition, the worms looked more active and the ability of the head-bulb and body to move appeared more quickly in BME. Moreover, the survival time of larvae in supplemented medium was longer than in the unsupplemented.

When 5% and 15% of heat inactivated (56 C 30 min) normal human serum were supplemented to the BME media, the mean survival period could be significantly increased to 54.66 and 55.09 days respectively. In this condition, some larvae remained alive for as long as two and a half months. It is possible that the enrichments are essential for worm survival in vitro. Similarly, the medium supplemented with purified bovine serum albumin (BSA, E. Merck, Rahway, Germany) showed that BSA was also effective in prolonging worm survival. Although the results presented in the Table indicate that the mean survival period in the media with 7.5 mg/ml BSA supplement was somewhat shorter than that with 2.5 mg/ml BSA supplement, it had no significantly different effect on worm survival.

Discussion

We show a possible method of cycling G.spinigerum in the laboratory. These detailed methods followed those of Daengsvang[10] Such system may be useful in the study of various aspects of the host-parasite relationship including immune responses. In our experiment, the L_3 that were harvested from the experimentally

infected mice could be mintained metabolically active in vitro for an extended period of time under the appropriate conditions. The ability of worms to survive could be enhanced when the well-defined BME medium was supplemented with some enrichment components, i.e., normal serum. The enrichment effect of serum could be partially replaced by a purified fraction of serum macromolecule, i.e., serum albumin.

Table Survival of L_3 G.spinigerum maintained in vitro

Medium*	Supplemented with	No. of worms	Day of survival	
			Range	Mean ± SE
NSS	-	10	9-28	17.60 ± 6.63
HBSS	-	21	20-60	34.14 ± 10.14
BME	-	90	19-62	36.41 ± 9.99
BME	5% Normal human serum▲	12	26-58	54.66 ± 9.05t
BME	15% Normal human serum▲	21	26-76	55.09 ± 10.05t
BME	2.5 mg/ml Bovine serum albumin	10	29-46	43.50 ± 5.19tt
BME	7.5 mg/ml Bovine serum albumin	11	31-48	41.18 ± 4.28tt

* Medium was changed every 48 hr.

▲ Serum was heated at 56 C 30 min

t Significantly different from unsupplemented BME (P-value < 0.001)

tt Significantly different from unsupplemented BME (P-value < 0.05)

Nevertheless, the final concentration of BSA used, although higher than that found in normal serum, led to an inferior enhancement than with whole serum. There may be some other required component (s) in serum. Moreover, all L_3 under our cultivation conditions did not undergo any morphological changes of their lips, head-bulb and body.

ES products showed enzyme-immuno reaction with serum from patients with Gnathostoma infection. These assays were tested with 8 patients (Data not shown) whose stool examinations were negative for other parasites. Six of them had clinical signs and symtoms of cutaneous gnathostomiasis and two had worms removed from their skin. Interestingly, one of these two, who had a worm removed from her skin 4 weeks after she had eaten uncooked and fermented fish, "Som-Fak", showed negative intradermal reaction to somatic L_3 crude antigen while the enzyme-immuno reaction with ES was many times more positive than the normal activity.

Our in vitro cultivation system may assist furthur research on Gnathostoma and can possibly be used to produce large

quantities of ES products which may express the potential or functional antigen for serodiagnosis as well as for vaccine production. In addition, the system may also be employed for the in vitro detection of potential anthelmintic drugs in the treatment of Gnathosotma infection.

Acknowledgements

The authors are grateful to Dr.Kampol Pecharanond for helpful discussions during the course of this study, his suggestions and criticiums during the preparation of this manuscript. Thanks must be given to Dr. Phisai Kraivichian, head of the Department of Parasitology Faculty of Medicine, Chulalongkorn University. This work was supported in part by the Rachadapiseksompoj China Medical Board Research Grant.

References

1. Daengsvang S. Gnathostomiasis in Southeast Asia. Southeast Asian J Trop Med Public Health 1981 Sep; 12(3) : 319-332
2. Daengavang S. Gnathostoma Spinigerum Owen; 1836. In : SEAMIC Southeast Asian Medical Information Center. A monograph on the genus Gnathostoma and Gnathostomiasis in Thailand. Tokyo, Japan, 1980, 4-47
3. Voller A, de Savigny D. Diagnostic serology of tropical parasitic disease. Immunol Methods 1981; 46(1) : 1-29
4. Egashira M. Studies on Gnathostoma spinigerum. A study on the diagnosis for human gnathostomiasis. Acta Med 1953; 23(1); 114-122 (in Jap. with Eng abstr.)
5. Pierre P, Luffau G. Antigens of Helminths. In: Sela M, ed. The Antigense. London: Academic Press, 1979. 83-172
6. Daengsvang S. Penetration of the skin by Gnathostoma spinigerum larvae. Ann Trop Med Parasit 1970; Sep 64(3) : 399-404
7. Sooksri V. Studies on different species of cyclops in Bangkok and neighbouring areas with reference to those acting as first intermediate host of Gnathostoma spinigerum. A thesis submitted to the Faculty of Graduate Studies University of Medical Sciences (now Mahidol University), 1967
8. Lowry OH, Rosebrongh NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275
9. Voller A, Bartlett A, Bidwell DE. Enzyme immunoassay for parasitic diseases. Trans R Soc Med Hyg 1976; 70(2) : 98-106
10. Daengsvang S. Gnathostoma spinigerum. J Parasit Trop Med Assoc Thai 1981; 4(2) : 53-63

จุฬาลงกรณ์เวชสารได้รับต้นฉบับเมื่อวันที่ 1 เดือน กรกฎาคม พ.ศ. 2528