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In vitro cultivation of *Gymnophalloides seoi* metacercariae (Digenea: Gymnophallidae)

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Abstract: *Gymnophalloides seoi* is a human intestinal trematode prevalent on southwestern islands in Korea. In the present study, we investigated whether *G. seoi* metacercariae can grow and develop into adults by *in vitro* cultivation. The metacercariae were obtained from naturally infected oysters, and cultured *in vitro* for 5 days under three conditions; 37°C/5% CO_2, 41°C/8% CO_2, or 41°C/5% CO_2, in NCTC 109 complete media containing 20% FBS and 1% antibiotics-antimycotics. The degree of worm growth and development was compared with that grown *in vivo* of C3H mice. The length of the worms cultivated *in vitro* was 200-300 μm, not significantly different from metacercariae, whereas the length of the worms recovered from C3H mice was significantly larger, 300-400 μm. The worms produced eggs when grown in C3H mice or cultured *in vitro* for 2 days under 41°C/8% CO_2 or 41°C/5% CO_2, but not when cultured under 37°C/5% CO_2. Among the *in vitro* conditions, 41°C/5% CO_2 was best for egg production, although the number of eggs was about half of worms obtained from C3H mice. In conclusion, *in vitro* cultivation of *G. seoi* metacercariae into egg-producing adults was partially successful under culture conditions of 41°C/5% CO_2 or 41°C/8% CO_2.

Key words: *Gymnophalloides seoi*, metacercaria, adult, *in vitro* cultivation, egg

INTRODUCTION

Gymnophalloides seoi Lee, Chai and Hong, 1993 (Digenea: Gymnophallidae) was described as a new human intestinal trematode in Korea (Lee *et al.*, 1993). High prevalence of human *G. seoi* infection was subsequently reported on a southwestern coastal island of Shinan-gun, Chollanam-do (Lee *et al.*, 1994). Human infection was also discovered in Muan-gun, Chollanam-do, 25 km northwards from Shinan-gun (Chai *et al.*, unpubl. obs.). There are many questions to be answered regarding the biological aspects of *G. seoi*. One of the essential points to be solved is to find out the complete life cycle of this trematode, especially the first intermediate host and natural final hosts other than humans and oystercatchers (Yang *et al.*, 1996).

Considerable numbers of adult flukes are needed for immunological studies including antigen preparation, as well as various biochemical or physiological studies on *G. seoi*. So far, adult worms have been chiefly obtained from experimental infection of C3H mice. However, the recovery rate of worms, unless the host animals were immunosuppressed, was unsatisfactory (Lee *et al.*, 1997). Therefore, *in vitro* cultivation of metacercariae

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should be helpful to obtain a great number of adult flukes.

Many workers have attempted to cultivate various species of trematodes, from metacercariae or juvenile flukes, to sexually mature adults *in vitro*, with some success (Silverman and Hansen, 1971; Taylor and Baker, 1987; Seo, 1989). Among the family Gymnophallidae, cultivation of *Parvatrema timondavidi* was tried, and a considerable degree of worm maturation was reported (Yasuraoka *et al.*, 1974). For this purpose, chemically defined media such as RPMI 1640, Eagle's medium, and NCTC 109, containing most of the essential amino acids, vitamins, and other nutrients essential for the growth of animal cells, have been employed (Smyth, 1990). This study was performed to explore a suitable condition for *in vitro* cultivation of *G. seoi* metacercariae into egg-producing adults using NCTC 109 medium.

MATERIALS AND METHODS

1. Source of metacercariae

The oysters, *Crassostrea gigas*, were purchased from Aphae-myon, Shinan-gun, the well known endemic area of *G. seoi* (Lee *et al.*, 1994). The oyster shell was removed, and the animal was slightly digested in artificial digestive juice (0.5% pepsin 1:10,000 in 0.6% HCl solution) at 37°C for 5 minutes. The digested material, which contained freed metacercariae, was washed several times with normal physiological saline. After repeated sedimentation and washing the metacercariae were collected under stereomicroscopy.

2. Sterilization of the metacercariae

The metacercariae isolated were washed rapidly several times with sterilized phosphate buffered saline (PBS, pH 7.2) containing double strength antibiotics, 400 units/ml penicillin and 2,000 μg/ml streptomycin.

3. *In vitro* cultivation

In a preliminary study, we tested RPMI 1640, Eagle's medium and NCTC 109 for cultivation of *G. seoi*. The results showed no significant difference among the three kinds of media (data not shown). Hence, NCTC 109 medium was chosen for further study.

A batch of about 60 metacercariae of *G. seoi* was placed in a 24-well plate (Nunc), each well containing 300 μl NCTC 109 medium (Gibco) supplemented with 20% fetal bovine serum (FBS), penicillin 200 units/ml, and streptomycin 1,000 μg/ml, which had been sterilized by filtration before use. Three different incubation conditions were prepared; 37°C/5% CO_2, 41°C/8% CO_2, and 41°C/5% CO_2. The medium was changed with a fresh one at intervals of 3 days by aspirating except for the layer of approximately 100 μl containing the flukes. Ten worms cultured at each condition were randomly selected, observed, and measured.

4. Experimental infection of C3H mice and recovery of worms

Young male C3H mice (20-30 g) were used for comparison with worm growth *in vitro*. The mice were infected each with 100 metacercariae of *G. seoi* using a gavage needle inserting into the stomach. In order to observe the maturation status of worms the infected C3H mice were sacrificed at day 2, 3 and 5 post-infection (PI). Worms were recovered using Baermann's apparatus (Beaver *et al.*, 1984). Ten worms were randomly selected for measurement of the worm dimension.

RESULTS

1. *In vitro* cultured worms

There was no recognizable growth and development of worms when they were cultured under the condition of 37°C/5% CO_2 (Fig. 1, 2), although over 95% of worms were alive until day 5 after culture. The average length of day 2 to day 5 worms was within the range of 241-284 μm (n = 10), which were not significantly different from that of metacercariae (av. 268 μm; range 235-300 μm) (Fig. 1). In worms cultured under this condition sexual maturation was not recognizable because of no eggs in their uteri (Fig. 2).

The length growth of worms cultured under the condition of 41°C/5% CO_2 or 41°C/8% CO_2 was also not remarkable (Fig. 1); 244-292 μm or 244-246 μm (average values) at day 2-5, respectively. Meanwhile, sexual maturation of

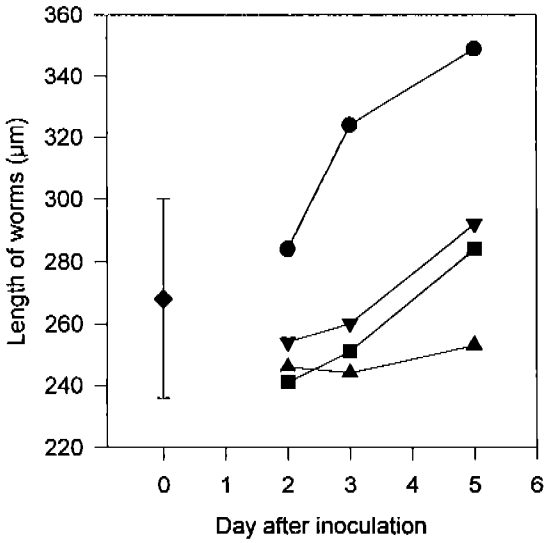

Fig. 1. The length growth (average values) of *G. seoi in vivo* and *in vitro*.

(◆) metacercariae (vertical bar indicates SD)
(●) *in vivo* of C3H mice
(■) at 37°C with a gas phase of 5% CO_2
(▲) at 41°C/8% CO_2
(▼) at 41°C/5% CO_2
Standard deviation (SD) never exceeded 25% of the average value.

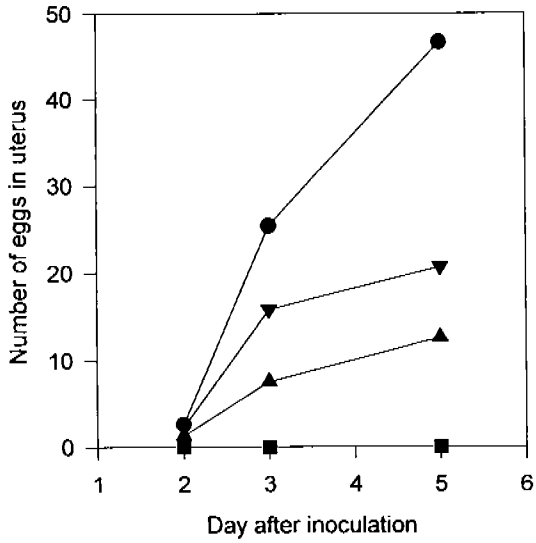

Fig. 2. Average number of uterine eggs in *G. seoi* grown *in vivo* and *in vitro*.

(●) *in vivo* of C3H mice
(■) at 37°C with a gas phase of 5% CO_2
(▲) at 41°C/8% CO_2
(▼) at 41°C/5% CO_2
Standard deviation (SD) never exceeded 25% of the average value.

these worms, particularly in terms of number of uterine eggs, was much better than those cultured at 37°C/5% CO_2 (Fig. 2). All of the reproductive organs were well developed in these worms, and a few eggs already appeared in the uterus on day 2 after culture.

So far as the number of uterine eggs was concerned, 41°C/5% CO_2 condition was found to be better than 41°C/8% CO_2 condition. Under the former condition, the average number of eggs per worm was 2.3 on day 2, 15.8 on day 3, and 20.7 on day 5 (Fig. 2). Under the latter condition, however, it was 1.3 on day 2, 7.5 on day 3, and 13.7 on day 5 (Fig. 2).

2. Worms recovered from C3H mice

The average (n = 10) length of *G. seoi* worms recovered from the intestine of experimentally infected C3H mice was 284 μm at day 2 PI, 324 μm at day 3 PI, and 349 μm at day 5 PI (Fig. 1), the latter two of which were significantly different ($p < 0.05$) from the size of metacercariae and worms cultured for 2-5 days *in vitro*.

The average (n = 10) number of uterine eggs in worms grown in C3H mice was 2.6 on day 2 PI, 25.4 on day 3 PI, and 46.7 on day 5 PI (Fig. 2). The latter two were also significantly different ($p < 0.05$) from worms cultured *in vitro* for 2-5 days under 41°C/5% CO_2 or 41°C/8% CO_2.

DISCUSSION

Metacercariae of *G. seoi* were successfully grown into adult flukes containing eggs in NCTC 109 medium supplemented with 20% heat-inactivated fetal bovine serum under a gas phase of 5% or 8% CO_2 in air at 41°C. However, the number of uterine eggs and size of worms cultured *in vitro* were significantly smaller than those grown *in vivo* of C3H mice, indicating that the *in vitro* conditions were less satisfactory for worms to mature than experimental infection to C3H mice.

It is well known that metacercariae of various kinds of trematodes including gymnophallids possess genital primordia such

as the ovary and testes so that they can develop early. Kannangara and Smyth (1974) noticed that trematode species possessing genital primordia produce eggs when stimuli such as exposure to the light or change of the temperature were given.

The role of animal serum for growth and/or maturation of worms has also been studied. An experiment with *Microphallus similis* supported evidence of spermatogenesis and vitellogenesis in culture medium not containing serum, *i.e.*, HBSS or NCTC 135 alone (Davies and Smyth, 1979). However, the worms thus cultured could not survive over 3 days and were obviously degenerating (Davies and Smyth, 1979). Therefore, sera seem not essential for sexual maturation of worms but indispensible as nutriments for survival and maintenance of worms.

There has been controversy whether heat inactivation of sera helps worm development/survival or not. Yasuraoka *et al.* (1974) reported that heat inactivated chicken and bovine sera were useful for growth and development of a gymnophallid, *Parvatrema timondavidi*, but unheated sera revealed inhibitory effect on the survival of worms. Similarly, Fujino *et al.* (1977) demonstrated that heat inactivated sera appeared effective for *in vitro* development of *Microphalloides japonicus*. On the other hand, NCTC 135 medium supplemented with heat inactivated sera showed inhibitory effect on development of *Amblosoma suwaense* to adults (Schnier and Fried, 1980). In the present study, however, heat inactivated sera were useful for maturation and development of *G. seoi*.

G. seoi did not produce eggs when cultured at 37°C with 5% CO_2 gas phase, while those cultured at 41°C with 5% or 8% CO_2 contained more or less number of eggs. Similarly, Yasuraoka *et al.* (1974) observed that *P. timondavidi* grew faster at 41°C than at 37°C, and when they were cultured at 41°C in NCTC 109 medium supplemented with 20% bovine or chicken serum, they grew at a rate approaching to that found *in vivo* of mice. Therefore, the temperature of 41°C seems adequate for development and sexual maturation of gymnophallids for *in vitro* cultivation.

The temperature preference of 41°C by *P. timondavidi* and *G. seoi* is well correlated with their natural life cycles; birds are taking the role for a natural final host (Yasuraoka *et al.*, 1974; Yang *et al.*, 1996). However, it should not be an essential condition for growth and maturation of *G. seoi*, considering that many humans, having 37°C body temperature, are infected with this fluke in endemic areas (Lee *et al.*, 1994), and C3H mice, also having 37°C body temperature, are a fairly good laboratory host (Lee *et al.*, 1997).

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=초록=

Gymnophalloides seoi (Digenea: Gymnophallidae) 피낭유충의 시험관내 배양

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참굴큰입흡충(*Gymnophalloides seoi*)은 새로운 인체 기생 장흡충으로서 최근 우리 나라에서 인체 감염의 유행이 보고된 바 있다. 이 연구는 참굴큰입흡충의 피낭유충을 시험관내에서 배양하여 성충으로 발육시킬 수 있는지 알아보고자 시행하였다. 피낭유충은 자연산 감염 굴에서 회수하였고, NCTC 109 배지에 우태아 혈청(FBS) 20%와 항생제-항진균제(Gibco) 1%를 첨가하여 사용하되 37°C/5% CO_2, 41°C/8% CO_2 및 41°C/5% CO_2의 3가지 배양조건 하에서 배양하였다. 배양 2일에서 5일까지 각 조건 하에서 얻은 충체를 실험 감염 C3H 마우스에서 회수한 충체와 발육 및 성숙도를 비교하였다. 배양군의 충체 크기는 길이 200-300 μm로서 피낭유충과 비교할 때 유의한 차이를 보이지 않았으나, 마우스로부터 회수한 충체는 300-400 μm로서 유의한 차이를 보였다. 마우스 체내, 41°C/5% CO_2 및 41°C/8% CO_2 조건 하에서는 모두 2일째에 충란이 형성되기 시작하였으나, 37°C/5% CO_2 하에서는 배양 5일까지도 충란이 형성되지 않았다. 배양군 중에서는 41°C/5% CO_2 조건이 충체 성숙 및 충란 생산에 가장 적합하였으나 마우스 체내에서 자란 충체에 비해 자궁내 충란 수가 약 1/2에 불과하였다. 결론적으로 참굴큰입흡충의 시험관내 배양은 41°C /5% CO_2 또는 41°C/8% CO_2 조건에서 부분적으로 성공적임을 알 수 있었다.

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