oncospheres needed more than 20 weeks to develop to maturity in SCID mice to be infective to both their natural and alternative definitive hosts.

Introduction

Asian taeniasis, caused by *Taenia saginata asiatica*, was first reported by Oi (1915) in central Taiwan . It has been reported in many other Asian countries such as several provinces in China, Korea, Indonesia, Thailand, Philippines, Malaysia and Myanmar (Fan et al. 1989, 1990a, 1992a, b; Eom and Rim 1993; Bowles and McManus 1994; Simanjuntak et al. 1997; Zhang et al. 1999; Fan 2000; Fan et al. 2001; Eom et al. 2002; Ito et al. 2003).

Compared with other human taeniids, the tapeworm of *T. s. asiatica* has no hook on its rostellum, which differs from *T. solium* but is similar to the classical *T. saginata* (*Taenia saginata saginata*), (Fan et al. 1995). However, in the intermediate host, metacestodes of *T. s. asiatica* parasitize the viscera, mostly in the liver, of pigs (Fan et al. 1995; Eom and Rim 2001), whereas *T. s. saginata* parasitize in the muscle of cattle (Schmidt and Roberts 2000).

Recently, eggs of *T. s. asiatica* have been reported to develop into mature cysticerci when injected into the subcutaneous tissue of severe combined immunodeficiency (SCID) and immunosuppressed ICR mice (Ito et al. 1997b; Ito and Ito 1999; Wang et al. 1999). The size of the cysticerci recovered from SCID mice was found to be larger than those found in pigs (Ito et al. 1997a). Moreover, 49% of the metacestode were seen to be calcified or degenerated in 34 infected pigs at 11–97 days post-infection, but no calcification of the metacestodes was observed in the SCID mice at 244 days post-infection (Wang et al. 2000; Fan et al. 1990c).

Since the SCID mouse has proved to be an experimental intermediate host model for the study of the development of *T. s. asiatica* metacestodes, it is necessary to confirm the infectivity of the cysticerci recovered from SCID mice. Humans are the only known natural definitive host of the three previously mentioned human taeniids. In addition, alternative definitive hosts have been used to test for the infectivity of the cysticerci. Alternative definitive host models for *T. solium* using golden hamsters, Mongolian gerbils, chinchillas and gibbons that were orally inoculated with cysticerci obtained from naturally infected pigs, have been reported. Gravid proglottids were recovered only from chinchillas and gibbons (Cadigan et al. 1967; Verster 1971, 1974; Maravilla et al. 1998). In *T. s. saginata*, sexually mature tapeworms were recovered from gerbils inoculated with cysticerci from naturally infected cattle (Kamiya et al. 1990). It has also been demonstrated that cysticerci of *T. solium* recovered from SCID mice could develop to maturity in hamsters,

although only pregravid tapeworms were observed (Wang et al. 1999). In the present study, we examined the infectivity and development of *T. s. asiatica* metacestodes from SCID mice in humans and its alternative definitive hosts -golden hamsters and Mongolian gerbils.

Materials and methods

Parasite

Proglottids of the *T. s. asiatica* adult worm were collected from a Taiwanese aborigine patient after deworming with atabrine (Quinacrine) (Fan et al. 1990b). The worms were stored at 4°C in saline until used.

Development of metacestodes in SCID mice

Eggs were collected from gravid proglottids. Embryophores were removed by incubating the eggs in 10% sodium hypochlorite for 10 min, and the oncospheres were washed five times in sterile saline. Eighteen SCID mice (C.B-17/Icr-scidJcl, female) purchased from a commercial source (CLEA, Tokyo, Japan) were subcutaneously inoculated with 20,000 and 40,000 oncospheres and intraperitoneally with 18,600 oncospheres of *T. s. asiatica*, respectively (Table 1). The SCID mice were kept in sterile cages and wood chips were used as bedding. The mice were provided with autoclaved drinking water and commercial pellet food (CLEA, Tokyo, Japan) ad libitum. All the inoculated SCID mice were anaesthetized under ethyl ether at 10, 20, 21, 24 and 45 weeks after inoculation. To check the evagination rate, cysticerci were incubated in calf bile in 37°C for 1 h. For morphological observation, evaginated cysticerci were stored in the refrigerator at 4°C in saline overnight, fixed in 70% alcohol, and cleared in glycerin.

[Table 1 will appear here. See end of document.]

Development of tapeworms in human and alternative definitive hosts

Three human volunteers (1 female, volunteer A, age 30, 2 males, volunteer B and volunteer C; age 26 and 51 years) each swallowed five 45-week-old cysticerci that were obtained from SCID mice. After ingesting the cysticerci, the volunteers checked their feces daily for the presence of proglottids. Fifty-five 3-week-old male golden hamsters were purchased from a commercial breeder (SLC, Shizuoka, Japan) and used for the experiment after 3 weeks, acclimatization (Table 2). The

hamsters were divided into two groups, designated H1 and H2, and then orally inoculated with 20 and 24 week-old cysticerci, respectively. Eighty-one 5–11 week-old male and female Mongolian gerbils raised in our laboratory, were used for the experiment (Table 2). The gerbils were orally inoculated with 10, 20, 21 and 45 week-old cysticerci and divided into 4 groups, namely G1, G2, G3 and G4. The animals were injected subcutaneously with prednisolone acetate at different schedules as shown in Table 2. They were then orally inoculated with cysticerci of *T. s. asiatica* obtained from SCID mice by stomach tubes and fed commercial pellet food (CLEA, Tokyo, Japan) and water *ad libitum*. Animals were anaesthetized with ethyl ether anaesthesia at 2 to 146 days post inoculation (DPI) and tapeworms collected from their intestines. After storing the worm in saline at 4°C overnight, the worms were fixed in 70% ethanol, stained with acid-carmine and observed under light microscope.

[Table 2 will appear here. See end of document.]

Results

Development of metacestodes in SCID mice

Metacestodes were recovered from the subcutaneous tissue inoculation site, as well as from the peritoneal cavity of SCID mice (Table 1). The recovery rates of metacestodes from intraperitoneally inoculated mice ranged from 0% to 0.3%, while those from subcutaneously inoculated mice ranged from 0.1% to 1.1% (Table 1, parasite source A). The recovery rate of metacestodes from subcutaneously inoculated mice was higher than those from intraperitoneally inoculated mice.

Evagination rate of the recovered cysticerci was 88% for 10 week-old cysticerci, 69% for 24 week-old cysticerci and 100% for 45 week-old cysticerci (Table 3). The total length of the evaginated cysticerci increased with age, as well as the average width of the scolex and diameter of the suckers (Table 3).

[Table 3 will appear here. See end of document.]

Microscopic observation revealed that calcareous corpuscles were few in 10 week-old cysticerci but abundant in 24 and 45 week-old cysticerci (Fig. 1b, 2b). Hooklets (rudimentary hooks) could be observed in 10 (Fig. 1c, d), 24 (Fig. 2c, d) and 45 week-old metacestodes (10–48% of the specimens all ages possess the hooklets). The rostellar region of 10 week-old cysticerci was full of small-granules aggregations (Fig. 1c, d), while the rostellar regions of 24 and 45 week-old cysticerci showed fewer small-granules aggregations (Table 3, Fig. 2c, d).

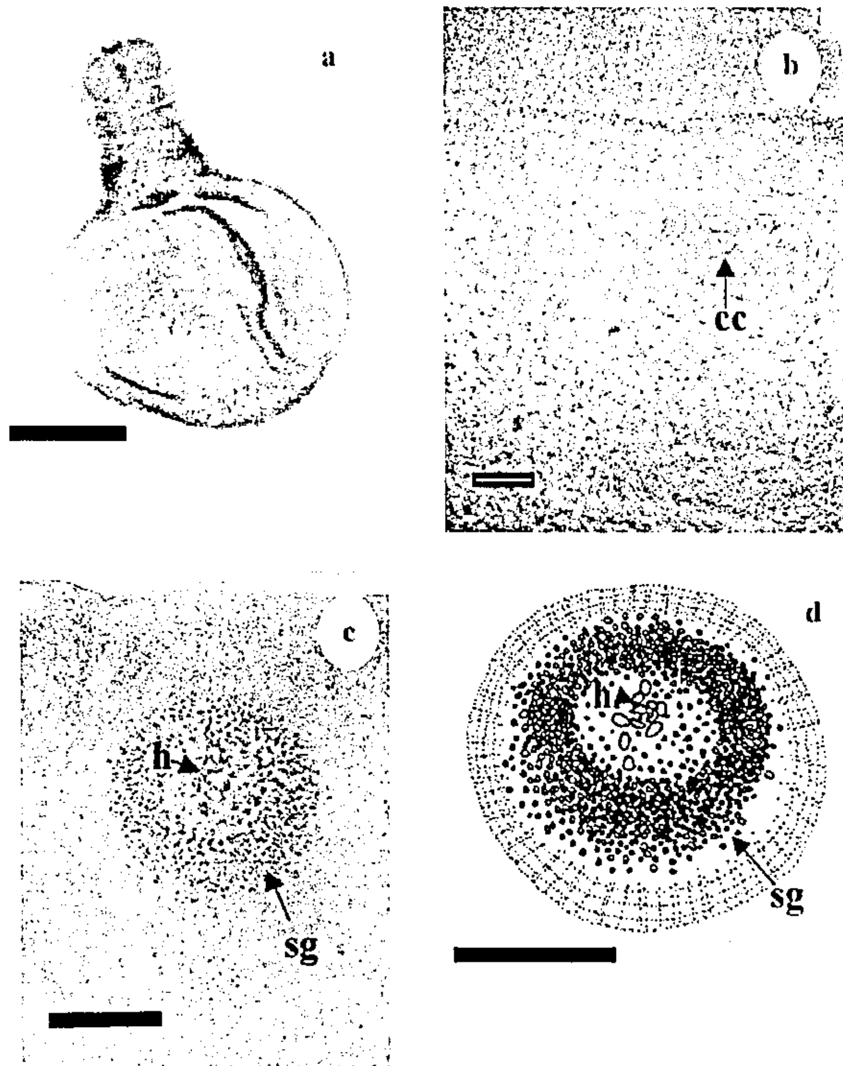

Fig. 1 **a–d** 10 week-old cysticerci recovered from SCID mice. **a** A whole specimen. *Bar*, 500 μm. **b** Few calcareous corpuscles (cc). *Bar*, 50 μm. **c** The rostellum. *Bar*, 50 μm. Hooklets (h) and small-granules aggregations (sg). *Bar*, 50 μm. **d** Drawing of the rostellum in the Fig. 1c

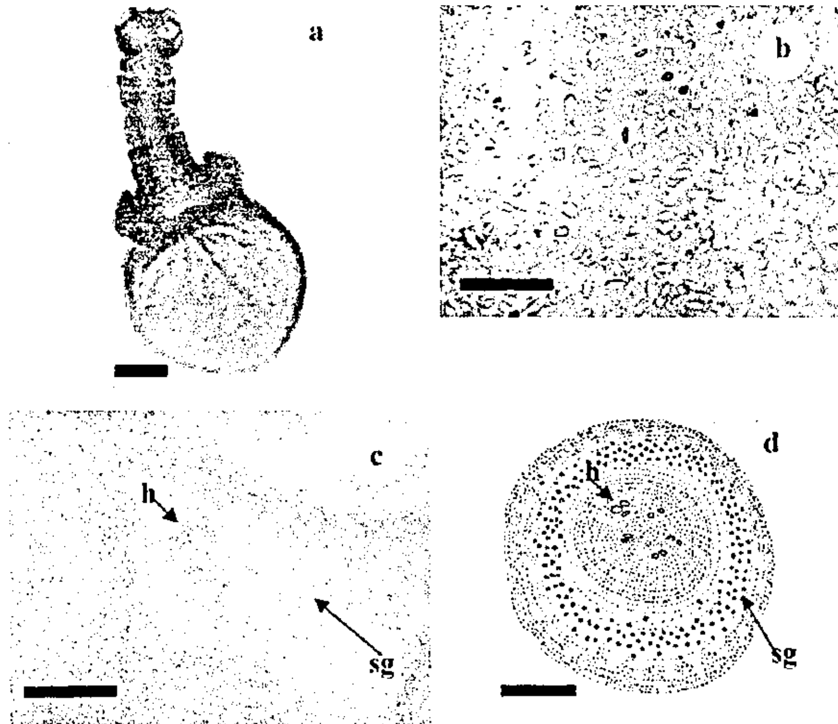

Fig. 2 a–d A 24 week-old metacestode recovered from SCID mice. Bar, 500 μm. **a** A whole specimen. Bar, 500 μm. **b** Calcareous corpuscles completely filled the neck region of the metacestode. *Bar*, 50 μm. **c**The rostellum. *Bar*, 50 μm. **d** Drawing of the rostellum in the Fig.1b. Hooklets (h) and small-granules aggregations (sg). *Bar*, 50 μm.

The recovery rate of tapeworms was 1.2% (3/246) for 20 week-old and 1.4% (3/210) for 24 week-old cysticerci in the golden hamster. The recovery rate of tapeworms were 0% (0/640) for 10 week-old, 1.0% (2/192) for 20 week-old, 0.5% (2/403) for 21 week-old and 5.8%(2/34) for 45 week-old cysticerci in Mongolian gerbils (Table 4).

[Table 4 will appear here. See end of document.]

Development of tapeworms in alternative definitive hosts

Tapeworms of *T. s. asiatica* were recovered from golden hamsters at days 6, 7, 12, 13 and 14 post-infection. The worms were also recovered from immunosuppressed gerbils at days 2, 7, 25, 36 and 62 post-infection (Table 4). Worms were detected in the anterior part of the small intestine. The length of the six tapeworms recovered from the six hamsters (one worm from each animal) after fixation, ranged from 1 mm to 6 mm, while that of the six tapeworms from the six gerbils ranged from 1 mm to 40 mm (Fig. 4). The tapeworm positive rate of the hamsters inoculated with 20 week-old cysticerci was 7% (3/41) and with 24 week-old cysticerci was 21% (3/14). The

tapeworm positive rate of the gerbils inoculated with 10 week-old cysticerci was 0% (0/21), with 20 week-old cysticerci in gerbils, 6% (2/32), with 21 week-old cysticerci, 18% (2/11), and with 45 week-old cysticerci in gerbils, 11% (2/17) (Table 4). Genital primordia were observed in posterior segments of the worms recovered from the gerbils on days 36 and 62 post-infection (Fig. 3). The longest worm (40 mm in total length) with 164 demarcated segments was recovered from a gerbil at 36 DPI.

Fig. 3 Tapeworm of *T. s. asiatica* recovered from a gerbil at day 36 post-infection. Genital primordia (g) in posterior segment of the strobila. Bar, 200 μm.

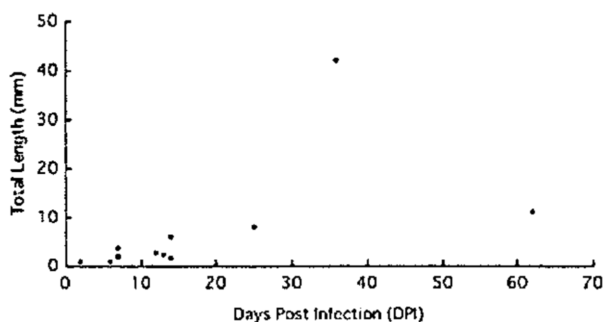

Fig. 4 Length of *T. s. asiatica* adult worm recovered from either gerbils (*black circle*) or hamsters (*white circle*) in the period of 2 to 62 days post-infection

Development of tapeworms in human

Two out of the three human volunteers who took the cysticerci shed strobila or segments of *T. s. asiatica* in their feces. Strobilae with gravid segments and without scolex were found on day 64 (1 strobila), 90 (3 strobilae) and 131 (1 strobila) post-infection from the volunteer A and on day 114 (2 strobilae), 138 (1 strobila) and 194 (1 strobila) post-infection from the volunteer B. Volunteer C did not shed any proglottids and was deemed not infected. The prepatent periods of *T. s. asiatica*

infection in the two human volunteers were 64 and 114 days, respectively. The two human volunteers were dewormed using atabrine at days 191 (expulsion of 3 strobilae) and 194 (expulsion of 1 strobila) post-infection, respectively. Gravid segment excretion of volunteer A was found from 81 to 90 (mean: 4.3 proglottids per day; ranging from 0 to 14) and from 177 to 190 (mean: 9 proglottids per day; ranging from 0 to 42) days post-infection. Volunteer B excreted gravid segments from 169 to 191 days (5.3 proglottids per day; ranged from 0 to 33) post-infection. Eggs were detected during and after the period of segment shedding in the feces.

Discussion

It has been reported that oral inoculation with eggs of *T. s. asiatica* could not produce successful infection in mice and that only intraperitoneal inoculation of oncospheres hatched in vitro could result in a high recovery rate of metacestodes from SCID mice (Ito et al. 1997a; Ito and Ito 1999). However, our results showed that only a few metacestodes could be recovered from intraperitoneally inoculated animals and the recovery rate was much lower than subcutaneously inoculated ones.

Metacestodes of *T. s. asiatica* had been found to develop to maturity in the liver of experimentally infected pigs, showing hooklets and active movement at 28 DPI (Fan 1988). In SCID mice, *T. s. asiatica* metacestodes had been reported to develop to cysticerci with a total length (after evagination) of 16 and 31 mm, and with suckers diameter of 199 and 252 μ m at 9 and 25 weeks after subcutaneous inoculation, respectively (Wang et al. 2000). We observed a similar increase in the total length of cysticerci, but the diameter of the suckers was larger than those reported by Wang et al. (2000). However, they also reported that the inner hooklets ranged from 11 to 19 and the outer hooklets were small and numerous on the rostellum of the 9 to 31 week-old cysticerci in addition to the numerous calcareous corpuscles in the neck region of the 9 week-old cysticerci (Wang et al. 2000). No further study on the differences of the number of the hooklets and calcareous corpuscles among the various ages of metacestodes had been published. In our study, the rudimentary hooks, reported to be inner hooklets by Eom and Rim (1993) and Fan et al. (1995), were observed in most but not all the cysticerci of all ages. We observed many small-granules aggregates in place of outer hooklets as reported by Eom and Rim (1993) and Fan et al. (1995) in the rostellar region, with few calcareous corpuscles in the 10 week-old cysticerci. In the 24 week-old cysticerci, we observed only few small-granules aggregates in the rostellar region but abundant calcareous corpuscles. We also demonstrated that the infectivity of the cysticerci recovered from SCID mice varied with age by using the alternative definitive host models. No adult tapeworm

could be recovered from the alternative definitive hosts (gerbils) inoculated with 10 week-old cysticerci, but several adult worms were recovered from the gerbils and hamsters orally inoculated with 20, 21, 24 and 45 week-old cysticerci. This could be due to the insufficient development of the 10 week-old cysticerci, whereas the older cysticerci were sufficiently developed to grow to maturity. Since the 45 week-old cysticerci showed the highest recovery rate among the various ages, they were thought to be more mature than other younger cysticerci.

However, in our study, animals inoculated with a higher dose of the 21 week-old cysticerci showed lower recovery rate than those inoculated with a lower dose of the 20 week-old cysticerci. It has been reported that golden hamsters inoculated with 20 cysticerci of *T. solium* showed a lower recovery rate than those inoculated with 10 cysticerci (Monroy-Ostria et al. 1993). The probability of competition for nutrient among the worms resulting in a high inoculation dose of cysticerci which led to a low recovery rate of adult worms should be considered.

Evagination of the cysticerci scolex in vitro by incubation with bile has been used for evaluating the viability of cysticerci (Peniche-Cardena 2002). Fan et al. (2000) showed that the evagination rate of the 9 week-old cysticerci recovered from SCID mice was 77% after incubation in 100% pig bile for 20 min at 37°C. However, the infectivity of those cysticerci has not been demonstrated. In our study, a high evagination rate (88%) was obtained for 10 week-old cysticerci but were found not to be infective to gerbils. Thus, a high evagination rate of the cysticerci does not always indicate high infectivity of the cysticerci in its definitive hosts. However, the presence of abundant calcareous corpuscles in the scolex and the degree of rostellar development could be considered as important criteria for evaluating the infectivity of the cysticerci.

Tapeworm recovery in the positive groups (H1, H2, G2, G3, and G4 groups) was 6.2% (2/32) at 1 to 7 DPI, 20.8% (5/24) at 8 to 14 DPI, 8.5% (3/35) at 15 to 28 DPI and 8.3% (2/24) at 29 to 62 DPI. The recovery rate of the latter phase (29 to 62 DPI) was not lower than that of the earlier phase (1 to 7 DPI). We hypothesize that tapeworms of *T. s. asiatica* could survive in the intestine of their hosts for a long period after their establishment there. Thus, a highly sensitive, reliable and quick detection method such as coproantigen ELISA test is needed to confirm the establishment and survival of the worms in the intestine of the alternative definitive hosts.

Proglottids were reportedly detected in one out of three human volunteers who had ingested 10 week-old cysticerci of *T. s. asiatica* obtained from the liver of an experimentally infected Freisian Holstein calf (Chao et al. 1988; Fan et al. 1987). The first gravid proglottid was found on day 122 post-infection (Chao et al. 1988). A Korean male volunteer showed that the first gravid proglottid of *T. s. a.* could be recoverable from feces 76 days after eating five cysticerci obtained

from naturally infected domestic pigs (Eom and Rim 1992). In our study, the first strobila shed with feces was observed at 64 DPI in the female volunteer, and 131 DPI in the male volunteer. Gravid proglottids were first observed at 81 in the former and 177 DPI in the latter. Thus, there is a variation in the prepatent period of human *T. s. asiatica* infection, but further study on the sex of the host is needed.

In the alternative definitive host infection, genital primordia were observed in the posterior segments of the tapeworms recovered on day 36 and 62 post-infection. It was obvious that *T. s. asiatica* develop better in the human host than in the alternative definitive hosts. However, a longer infection period might be necessary for their development in the alternative definitive hosts. In this study, the alternative definitive models were shown to be effective in demonstrating the infectivity of the various ages of *T. s. asiatica* metacestodes.

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Table 1 Recovery rates of *T. s. asiatica* metacestodes from eighteen SCID mice subcutaneously inoculated with oncospheres

Oncospheres			SCID mice		Metacestodes recovery		
Source[a]	Inoculation route	Inoculation dose	Number	Age of autopsy (weeks)	Number		Recovery rate (%)
					Average	Range	
A	Subcutaneous	20,000	5	12, 20	133.6	20–218	0.1–1.1
	Intraperitoneal	18,600	5	12, 20	10.8	0–50	0–0.3
B	Subcutaneous	40,000	3	24, 62	153.6	1–450	0.003–1.1
	Subcutaneous	20,000	5	10–45	814.2	174–2,000[b]	0.9–6.4

[a] Source A: eggs collected from worms after deworm treatment in December, 2003 and inoculated into mice on January 9, 2004
[a] Source B: eggs collected from worms after deworm treatment in April, 2004 and inoculated in May, 2004
[b] The number of recovered metacestodes 2000 metacestodes were recovered from 1of the 5 mice was estimated by the dilution method, and other samples were counted without dilution

Table 2 Alternative definitive host groups and their treatment

Animals				Cysticerci		Schedule of PA treatment[c]
Group	Number	Age (weeks old)	Sex[a]	Number[b]	Age (weeks old)	
H1	10	6	M	6	20	A
	10	6	M	6	20	B
	11	6	M	6	20	C
	10	6	M	6	20	D
H2	8	6	M	15	24	A
	6	6	M	15	24	D
G1	21	5–7	M, F	20–40	10	A
G2	11	6–9	M, F	6	20	A
	11	6–9	M, F	6	20	C
	10	6–9	M	6	20	D
G3	11	6–12	M	25–40	21	A
G4	11	6–11	M	2	45	E
	6	5	M	2	45	E

[a] *M* male; *F* female.
[b] Number of cysticerci/animal
[c] Dosage of PA (prednisolone acetate) was 0.2–0.5 mg per animal. PA treatment schedules are as follows: *A* every 7 days from 17–3 days before infection; *B* every 4 days from 6 days before infection; *C* every 2 days from 6 days before infection; *D* no PA treatment; *E* every 7 days from 7 days to 0 days before infection and every 14 days from 56 days after infection.

Table 3 Evagination rates and morphological measurements of *T. s. asiatica* cysticerci recovered from three SCID mice

Age of metacestodes (weeks)	Morphological observation of cysticerci					Evagination rate[a]
	Number of examined	Total length (μm) (Mean±SD)	Width of scolex (μm) (Mean±SD)	Diameter of suckers (μm) (Mean±SD)	No. of calcareous corpuscles	
10	n=24	1,750±470	590±130	220±60	Few (less than 50)	50/57 (88%)
24	n=30	3,890±800	680±100	290±30	Abundant	34/49 (69%)
45	n=9	5,930±870	710±60	300±40	Abundant	25/25 (100%)

[a] Evagination rate of cysticerci was examined in 100% calf bile at 37°C for 1 h

Table 4 Recovery of *T. s. asiatica* tapeworms from Mongolia gerbils and golden hamsters orally inoculated with cysticerci obtained from SCID mice

Animal	Cysticerci inoculated		Weeks post-infection at necropsy					Tapeworms recovery
Group	Age (wk)	Number[a]	<1 week	1-2 weeks	2-4 weeks	>4 weeks	Total number[b] (%)	Number[c] (%)
H1	20	6	0/16[d]	2/12	1/13	–	3/41 (7)	3/246 (1.2)
H2	24	15	1/1	1/1	1/6	0/6	3/14 (21)	3/210 (1.4)
G1	10	20, 40	–	0/21	–	–	0/21 (0)	0/640 (0)
G2	20	6	1/12	1/7	0/13	–	2/32 (6)	2/192(1.0)
G3	21	25-40	0/1	1/3	1/3	0/4	2/11 (18)	2/403 (0.5)
G4	45	2	0/2	0/1	-	2/14	2/17 (11)	2/34 (5.8)

[a] Number of inoculated cysticerci/animal
[b] Only one worm was detected in each infected host
[c] The number of tapeworms recovered from hamsters or gerbils/the number of cysticerci inoculated
[d] No. of positive animals/No. of examined

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Original Paper

Modified cellular immune responses in dogs infected with *Echinococcus multilocularis*

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Abstract Parasite-specific antigen responses and lymphocyte blastogenesis in dogs orally inoculated with *Echinococcus multilocuralis* metacestodes were examined. Serum IgG1 (Th2-oriented) and IgG2 (Th 1-oriented) levels against somatic and excretory-secretory (ES) antigens of protoscoleces and adult worms increased from 7 days post-infection (DPI), with the highest responses against protoscolex excretory-secretory antigen (PES). Specific blastogenesis of peripheral blood mononuclear cells (PBMC) against the parasite antigens was not observed during the 21-day infection period, but Peyer's patches cells from one out of two dogs at 21 DPI showed blastogenesis against PES (stimulation index: 4.65). Interestingly, only at 7 DPI were concanavalin A (ConA)-induce proliferative responses of PBMC reduced. Moreover, ConA-induced proliferative responses of lymphocytes from various origins were suppressed by the addition of parasite antigens, especially with PES. These data suggest that although both Th1- and Th2-oriented humoral immune responses were observed in *E. multilocularis* infected dogs, the parasite antigens, especially PES, may have incompletely suppressed lymphocyte responses in these dogs.

Introduction

Echinococcus multilocularis is the causative agent of a parasitic zoonosis, alveolar hydatidosis. The life cycle exploits a prey–predator relationship (voles as the intermediate hosts and carnivores as the definitive hosts), and human cases are caused by accidental ingestion of parasite eggs. A countermeasure against the source of infection (parasite eggs)

has been successfully developed by baiting wild foxes with the anthelmintic praziquantel (Schelling et al. 1997; Tackmann et al. 2001; Tsukada et al. 2002; Hegglin et al. 2003). Compared to deworming, immunoprophylaxis of the definitive hosts would have more advantages for a long-lasting preventive effect.

Although some evidence suggests the existence of protective immunity to *Echinococcus* in the definitive hosts both in experimental infections (Herd et al. 1975; Gemmell et al. 1986; Heath 1986) and field studies (Tackmann et al. 1998; Morishima et al. 1999; Yimam et al. 2002), the mechanisms have not yet been fully elucidated. Despite the need, little information is available on immunity to *Echinococcus* infections in definitive hosts because of its biohazardous nature and animal welfare problems. Although there are some works on the humoral and cellular immunity to *E. granulosus* infection, little work has been done on *E. multilocuralis* infection. In dogs infected with *E. granulosus*, at 35 days post-infection (DPI), parasite specific blastogenesis of local lymph node cells (mesenteric, popliteal, and Peyer's patches) were obtained from some dogs, and those of peripheral blood mononuclear cells (PBMC) resulted in low levels in a subset of dogs (Deplazes et al. 1994). According to Al-Khalidi and Barriga (1986), *E. granulosus* infection at 29 DPI depressed the ability of non-stimulated PBMC to proliferate, but enhanced concanavalin A (ConA)-stimulated proliferative responses. In contrast, many workers reported suppressed ConA- and antigen-induced splenocyte proliferation in the intermediate hosts of *Echinococcus* (Rakha et al. 1991; Kizaki et al. 1991; Dai and Gottstein 1999; Dematteis et al 2001).

Thus, it is hypothesized that the immune system, especially the intestinal immune system of the definitive host, may be stimulated by *E. multilocuralis* infection. To clarify this, antibody and lymphocyte responses to primary infection of *E. multilocularis* in dogs were examined. In addition, the effect of parasite antigens on ConA-induced lymphocyte proliferative responses was examined to assess immune modulation by parasite antigens.

Materials and methods

Parasites

The parasite used in this study was the 'Eiken' strain of *E. multilocularis*, originally obtained from a red backed vole (*Clethrionomys rufocanus bedfordiae*) in Nemuro, Hokkaido, Japan in 1986 and kept by dog–cotton rat (*Sigmodon hispidus*) cycles in the Hokkaido Institute of Public Health. Thereafter, this strain was maintained in Mongolian gerbils (*Meriones unguiculatus*) by serial passages of metacestodes (i.e., secondary hydatidosis) in our laboratory.

Animals and experimental infections

Two beagle dogs (male, 16 months old), kindly provided by the New Drug Development Research Center (Iwamizawa, Hokkaido, Japan), were kept in our laboratory and supplied with commercial dog food (CD-5, CLEA Japan, Tokyo, Japan) and water ad libitum. For initiating experimental infections in dogs, the *E. multilocuralis* metacestodes of 8 months post-infection were recovered from a gerbil, and the dogs were orally given about 230,000 protoscoleces. Then the dogs were bled on 0, 3, 7, 10, 14, 17 and 21 DPI. Peripheral blood was utilized for lymphocyte proliferation tests. Sera were also separated from the blood and

stored at –40°C until used for antibody detection. Feces were collected everyday from 7 days before infection and stored at –40°C until needed.

E. multilocularis coproantigen detection

Excretion of *E. multilocularis* coproantigen was monitored by the previously reported sandwich enzyme-linked immunosorbent assay (ELISA). The assay using a murine monoclonal antibody directed against somatic antigens of adult *E. multilocularis*, EmA9 (Kohno et al. 1995), was performed according to the method described by Morishima et al. (1999).

Antigen preparation

Metacestodes were recovered from gerbils and protoscoleces were isolated according to the method of Smith and Davis (1975). Adult worms (21 days old) were obtained from the rodent alternative definitive hosts described by Kamiya and Sato (1990) with a minor modification: prednisolone acetate (Predonine, Shionogi, Japan) was used in place of prednisolone tertiary butylacetate (PTBA).

To prepare somatic antigens of protoscoleces and adult worms (called PSO and ASO, respectively), isolated protoscoleces or adult worms were mixed with phosphate buffered saline (PBS, pH 7.4) containing 1% deoxycholic acid (DOC), 5 mM iodoacetamide, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μM pepstatin, disrupted first by homogenization using a glass homogenizer, then by sonication (30 W), and finally by repeated freeze-thaw lysis. Then, the suspension was subjected to centrifugation at 10,000 *g* for 30 min at 4°C. The resulting supernatant was dialyzed against PBS and used as a somatic antigen.

To prepare excretory-secretory (ES) antigens of protoscoleces and adult worms (called PES and AES, respectively), live protoscoleces or adult worms were incubated at 37°C in a 5% CO_2 incubator with Medium 199 (Gibco BRL, USA) supplemented with penicillin (100 IU/ml) and streptomycin (1 mg/ml), glucose (4.5 mg/ml), and KCl (0.2 mg/ml) (pH 7.4). Then, culture supernatant was filtrated through a 0.2 μm pore size filter (Advantec Toyo, Japan), placed in cellulose tubes with a cutoff molecular weight of 12,000–14,000 (Sanko Junyaku, Japan), concentrated using polyvinylpirrolidone (PVP-360 Sigma, USA), and then dialyzed against PBS. After dialysis, antigen solution was concentrated again with Centriprep Ym-10 or Ultrafree-15 Centrifugal Filter Device BioMax 10 K (Millipore, USA). Protein concentration of each antigen was determined by using the Bio-Rad Protein Assay Kit I (Bio-Rad, USA).

Parasite-specific antibody detection

E. multilocularis specific antibodies (serum IgG1 and IgG2, IgA, and fecal IgA) were detected by ELISA. Antigens and their concentrations utilized for plate coating were as follows: PSO (5 μg/ml), PES (0.5 μg/ml for IgG antibodies, 1 μg/ml for IgA antibodies), ASO (5 μg/ml), and AES (0.5 μg/ml for IgG antibodies, 1 μg/ml for IgA antibodies). After coating the plate with 50 μl of antigens in 0.05 M $NaHCO_3$-Na_2CO_3 buffer (pH 9.6)

overnight at 4°C, the plate was washed with PBS containing 0.05% Tween 20 (PBS-T) three times and blocked for 1 h with 100 μl/well of 1% bovine serum albumin (BSA, fraction V, Sigma, USA) in PBS. Then, the plate was washed with PBS-T three times and incubated either with 50 μl/well of sample sera or fecal supernatant for 1 h, all of which were assayed in duplicate. Sera were diluted 100-fold with PBS. Feces (10 g) were mixed with three times the volume (v/w) of PBS-T containing 0.02% sodium azide. The mixture was spun at 20,000 *g* for 15 min at 4°C and the resulting supernatant was diluted again twofold with PBS-T and used for antibody detection. After incubation of the plate with samples, the plate was washed again and incubated either with 50 μl/well of horseradish peroxidase (HRP)-conjugated goat anti-dog IgG1 (γ1) (diluted 1:10,000 in 0.5% BSA-0.5% casein in PBS-T), HRP-conjugated sheep anti-dog IgG2 (γ2) (1:10,000) or HRP-conjugated goat anti-dog IgA (α) (1:1,000) (all products from Bethyl Laboratories, USA) for 1 h. Then the plate was washed again and incubated with 100 μl/well of 0.04% *o*-phenylenediamine (Sigma) and 0.02% H_2O_2 in 100 mM citric phosphate buffer (pH 5.0) for 30 min at 37°C. Finally, 4 N H_2SO_4 was added to stop the reaction and the optical density (OD) at 490 nm of each well was read using an ELISA plate reader (Microplatereader, Model 450, Bio-Rad). The cut-off value of the ELISA for specific antibody detection was determined by calculating the mean+3SD of the samples collected before infection.

Lymphocyte proliferation tests

Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque-1077 (Sigma Diagnostics, USA) according to the manufacturer's instructions. At 21 DPI, dogs were sacrificed by injecting an overdose of sodium pentobarbital (Nembutal, Dainippon pharmaceutical, Japan), the spleen, mesenteric lymph nodes (MLN), and Peyer's patches (PP) were removed and lymphocytes were isolated from each organ. Single cell suspensions were prepared in RPMI 1640 (Gibco BRL, USA) supplemented with 10% inactivated fetal calf serum (FCS, Cosmo Bio, Tokyo, Japan), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol (Sigma, USA). Cell viability was assessed using 0.5% trypan blue (Wako Junyaku, Japan) in PBS. Then, cells (2×10^5 cells/100 μl/well) were cultured either with PSO (50 μg/ml final concentration) or PES (5 μg/ml) in 96-well culture plates (Corning, USA). Cells were also cultured with ConA (type IV, Sigma, USA) (5 μg/ml) for a positive control of cell reactivity or without stimuli. In addition, cells were co-stimulated with ConA and one of the parasite antigens. The cells were cultured at 37°C in a 5% CO_2 incubator for 72 h. The degree of cell proliferation was determined by a colorimetric MTT assay (Mosmann 1983) and the color development was measured at 570 nm. Stimulation indices (SI) were expressed as the ratio of the proliferation of stimulated cells to that of non-stimulated cells. An SI ≥ 2 was considered as significant (Gottstein et al. 1990).

Results

E. multilocularis coproantigen detection

During the course of experimental infection, excretion levels (OD values) of coproantigen from both dogs increased at 7 DPI and remained high until the end of the experiment (21 DPI) (Fig. 1). This indicates that *E. multilocularis* was successfully established in the intestine of the dogs during the experiment.

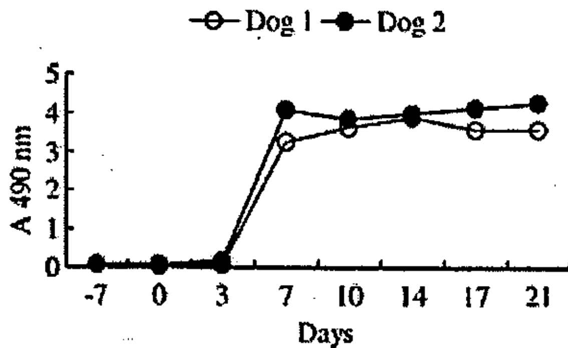

Fig. 1 Time course of *Echinococcus multilacularis* coproantigen detection in dogs. Dogs were orally infected with *E. multilacularis* metacestodes (containing 230,000 protoscoleces). *E. multilacularis* coproantigen was measured by sandwich ELISA using monoclonal antibody EmA9

Parasite-specific antibody detection

Serum antibody levels to *E. multilocularis* antigens are shown in Fig. 2. Serum IgG1 levels specific to four *E. multilocularis* antigens increased from 10 to 14 DPI, with the highest response observed with PES. In contrast, serum IgG2 levels increased earlier from 7 DPI, with the highest response observed with PES. An increase in the serum IgA level was observed in dog 2, with the highest response obtained with PES, while the increase was not obvious in dog 1. Parasite-specific fecal IgA titers showed high OD values before infection and high variation of daily OD values after infection.

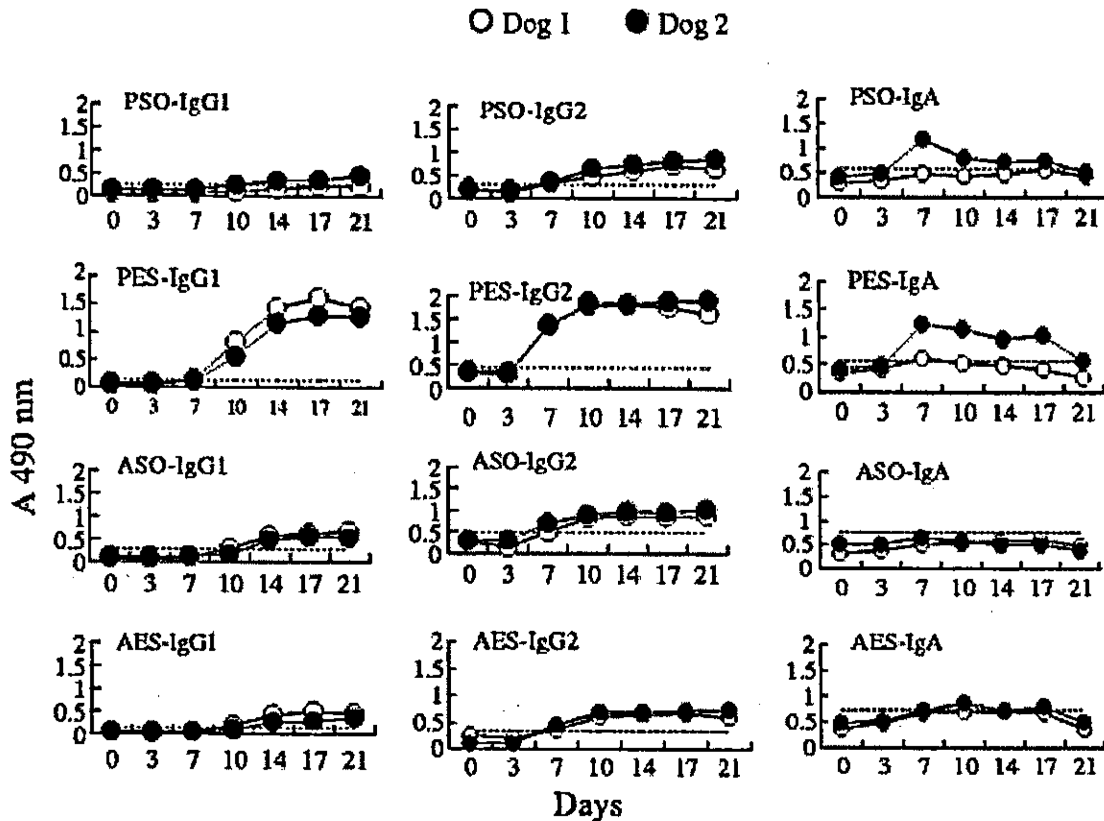

Fig. 2 Time course of serum antibody levels in dogs infected with *E. multilocularis*. *E. multilacularis* antigen specific IgG1, IgG2, and IgA levels were measured by ELISA. Data are presented as individual samples. Each sample assay was performed in duplicate. *PSO* Protoscolex somatic antigen, *PES* protoscolex excretory-secretory (ES) antigen, *ASO* adult worm somatic antigen, *AES* adult worm ES antigen. *Broken lines* indicate the cut-off values (the mean OD values +3SD of sera before infection)

Lymphocyte proliferation tests of PBMC

During the course of infection, responses of PBMC against ConA at 7 DPI decreased remarkably, but were restored thereafter (Fig. 3). Responses of PBMC against PSO and PES were not remarkably different from those of non-stimulated cells throughout the infection. Co-stimulation of PBMC with ConA and the protoscolex antigens, particularly with PES, resulted in lower responses compared with the responses with ConA mono-stimulation (maximum 60.7% reduction in dog 1 at 7 DPI).