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Citation	Japanese Journal of Veterinary Research, 44(1), 1-12
Issue Date	1996-05-31
DOI	https://doi.org/10.14943/jjvr.44.1.1
Doc URL	https://hdl.handle.net/2115/2520
Type	departmental bulletin paper
File Information	KJ00002398202.pdf

PARENTERAL STROBILAR DEVELOPMENT OF *ECHINOCOCCUS MULTILOCULARIS* IN SCID MICE

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ABSTRACT

Parenteral strobilation of *Echinococcus multilocularis* was observed in scid (severe combined immuno-deficient) mice after intracerebral, subcutaneous and intraperitoneal inoculation with protoscoleces. Evaginated protoscoleces and segmented worms were obtained at the inoculated sites. Most worms recovered from peritoneal cavity of scid mice were encapsulated by connective tissue and granulocytes but showed a maximum of 3 proglottids, elongation of genital primordia and vesiculation. Viability of worms recovered from the subcutaneous tissue and peritoneal cavity of scid mice were higher (69.1–91.4%) than those from the immunologically normal C.B-17 (4.0–48.0%) control mice. However, viabilities of worms from the cerebrum of both scid and C.B-17 were almost the same (87.7–94.4%). Worms recovered from scid mice showed further development of reproductive organs when transplanted into the small intestine of prednisolone treated golden hamster. These findings suggest that the parenteral milieu of scid mice allows adult development of *E. multilocularis* protoscoleces.

Key Words : *Echinococcus multilocularis*, scid mice, strobilation, parenteral development

INTRODUCTION

Some taeniids such as *Echinococcus granulosus, E. multilocularis* and *Taenia crassiceps* are known to undergo asexual reproduction in their intermediate hosts[11]. However, metacestode of these aforementioned taeniids has been observed to possess a dual developmental potential; when ingested by canids, they develop to adult tapeworm and undergo sexual reproduction in the small intestine, but when inoculated

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parenterally into an intermediate host, they develop asexually at the inoculation site. Strobilation of *T. crassiceps* metacestode has been observed in the intraperitoneal cavity of experimentally infected Mongolian gerbils[8]. Somatic growth of *E. multilocularis* protoscoleces has also been reported in the trachea of prednisolone treated golden hamsters and also in mice[15]. Although the ability of *E. multilocularis* protoscoleces to strobilate and thus initiate development toward the adult tapeworm stage in parenteral environment has been observed, factors regulating this development have not been studied.

The scid (severe combined immuno-deficient) mouse arose from a mutation on chromosome 16 in C.B-17 mouse, a BALB/c congenic strain, leading to a defect in V(D)J recombinase, and thus resulting in a deficiency in functional T and B lymphocytes[2]. Although the scid mice are hypogammaglobulinemic, their monocytes, granulocytes, erythrocytes and natural killer cells are normal[3-5]. We report herein the parenteral development of *E. multilocularis* protoscoleces in an environment devoid of functional lymphocytes as offered by scid mice.

MATERIALS AND METHODS

Animals

Seven-month-old and 7–8 week-old female scid mice were used in the first and second experiments respectively. C.B-17 mice of similar age were used as controls. The mice, obtained from the Central Institute for Experimental Animals, Kawasaki, Japan, were kept in filter-topped metal cage and maintained under specific free condition at 24°C with a 12 hour dark-light cycle. They were given commercial pellet food (CE–2, Clea Japan Inc., Tokyo) and sterilized water *ad libitum*.

Three-month-old female CN inbred golden hamsters were obtained from Nippon Institute for Biological Science, Tokyo, Japan. They were treated with prednisolone tertiarybutylacetate (PTBA, Merck & Co., Rahway, U. S. A.) according to the procedure described by Kamiya and Sato (1990)[7]. The hamsters were maintained as described for the mice except that they were kept under conventional condition.

Parasite and infection protocol

E. multilocularis used in this study was originally isolated from Bedford's red-backed voles (*Clethrionomys rufocanus bedfordiae*) in Nemuro, Hokkaido in 1987 and had been passaged at Hokkaido Institute of Public Health, Sapporo, Japan, using dogs and cotton rats (*Sigmodon hispidus*). Protoscoleces that were used in our experiments were passaged from a cotton rat into Mongolian gerbil (*Meriones unguiculatus*) for one generation. Larval cysts from the gerbils were collected in sterile physiological saline from the peritoneal cavity, minced and filtered through a 200 μm pore size metal mesh to obtain the protoscoleces, which were washed 3 times with saline. Their viability as determined by staining with 0.25% trypan blue was found to be 98% and 97% were not evaginated. Inoculation of the parasite was done on animals under

diethyl ether anesthesia.

In the first experiment, each of 16 scid and 8 C.B-17 mice were simultaneously inoculated intracerebrally with 1000 protoscoleces, and intraperitoneally and sub-cutaneously with 10,000 protoscoleces.

In the second experiment, 8 scid mice were intraperitoneally inoculated with 30,000 protoscoleces. In addition, approximately 1,500 worms recovered from the scid mice at 7 weeks post-inoculation (PI) were surgically transplanted into the small intestines of 3 golden hamsters.

Worm recovery and histopathological examination

In the first experiment, 6 scid and 4 C.B-17, 5 scid and 1 C.B-17, and 5 scid and 3 C.B-17 mice were killed, under diethyl ether anaesthesia, at 2, 4 and 6 weeks PI, respectively. In the second experiment, 5, 2 and 1 scid mice were killed at 7, 9 and 12 weeks PI, respectively. To recover the worms, the tissue inoculated with proto-scoleces were removed separately and a portion fixed in 10% phosphate-buffered formalin for histopathological examination. The remaining tissues were macerated in saline and the number of worms counted under a dissection microscope.

Golden hamsters that were transplanted with worms from scid mice were killed on day 8 post-transplantation. Their small intestine were removed and the intestinal mucosa scraped and fixed in hot 10% formalin. Worms were then collected from the preparation under a dissection microscope.

Tissue fixed in phosphate-buffered formalin was dehydrated in an alcohol series, embedded in paraffin and sectioned at 4 μm. The sections were either stained with haematoxylin and eosin (HE) or with Periodic acid Schiff (PAS) stain, and examined under a light microscope.

Viability of the worms was expressed at the percentage of worms that were not stained by 0.25% trypan blue solution. A portion of the recovered worms was fixed in 70% alcohol immediately after necropsy and the percentage of evaginated or strobilated worms determined. At least 100 worms were examined for each sample. For measuring the body length, the worms were left to relax in cold water and then fixed in 70% alcohol followed by staining with acetocarmine and observed under light microscope.

RESULTS

Experiment 1.

Body length of worms recovered from all inoculated sites of scid mice and cerebrum of C.B-17 mice increased along with the course of infection. However, no significant increase was observed in the length of worms recoverd from the sub-cutaneous tissue and peritoneal cavity of C.B-17 mice (Fig. 1).

Strobilation of the worm from scid mice was observed from 4 weeks PI but the percentage of strobilated worms was only 1–2% of the worms recovered.
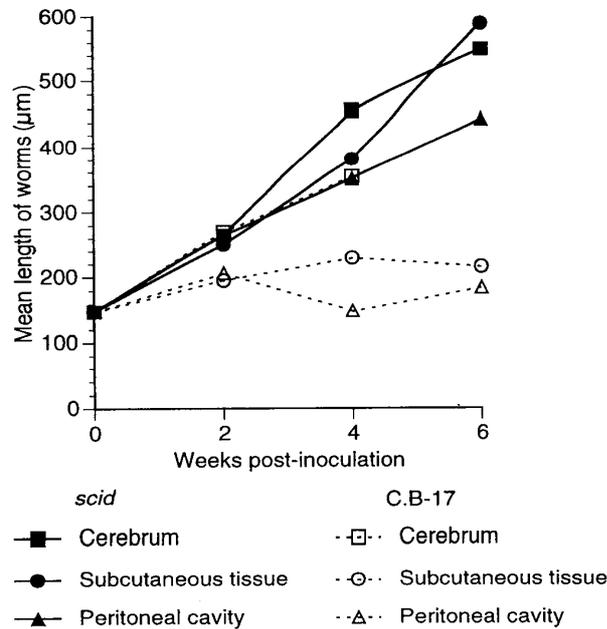
Fig. 1. Mean length of *E. multilocularis* worms recovered from scid and
C.B-17 mice after parenteral inoculation with protoscoleces.

Viability of the worms recovered from the subcutaneous tissue and peritoneal cavity of scid mice (69.1–91.4%) was higher than those from the C.B-17 mice (4.0–48%). However, viability of worms from the cerebrum of both scid (90.2–94.3%) and C.B-17 (87.7–94.4) were almost the same (Fig.2).

Evaginated protoscoleces were recovered from all inoculation sites of both scid and C.B-17 mice at 2, 4 and 6 weeks PI. The average percentage of evaginated protoscoleces among the recovered worms was 95% for scid and 68% for C.B-17 mice.

Evaginated protoscoleces recovered from subcutaneous tissue and peritoneal cavity of scid were encapsulated with a comparatively thin connective tissue layer (Fig. 3) and cellular infiltration consisting mainly of neutrophils, eosinophils and fibroblasts (Fig. 4). In contrast, those larval cyst mass recovered from the C.B-17 mice were encapsulated with a thick connective tissue layer and many granulocytes. The center of the cyst contained necrotic mass with degenerated worms, neutrophils and some PAS-positive fragments which were thought to be the laminated layers of the worm (Fig. 5 & 6). However, evaginated protoscoleces from the cerebrum of both the scid and C.B-17 mice were not encapsulated.

Experiment 2.

Two different types of larval cyst mass, namely hard and soft cysts, were recovered from the peritoneal cavity of scid mice at 7 weeks PI. As the course of
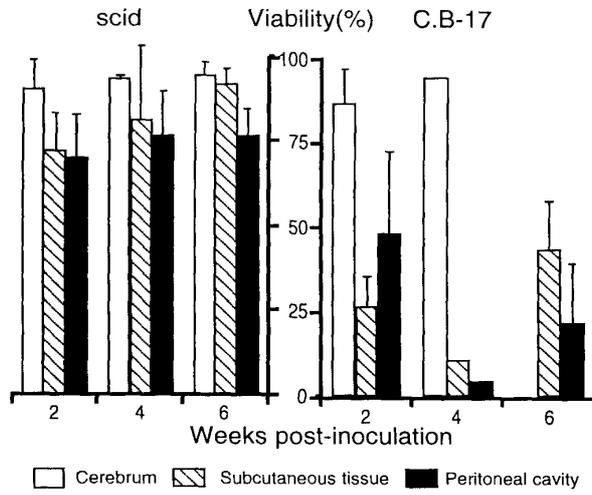
Fig. 2. Viability of *E. multilocularis* worms recovered from scid and C.B-17 mice after inoculation with protoscoleces at different sites. Viability was expressed as the percentage of worms which were not stained by trypan blue.
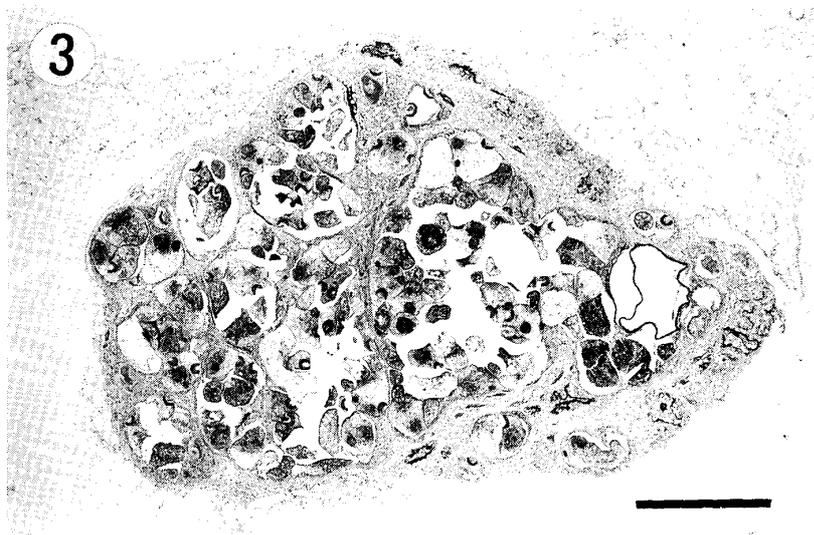

Fig. 3. *E. multilocularis* in subcutaneous tissue of scid mouse at 4 weeks post-inoculation. Many evaginated protoscoleces could be seen encapsulated in connective tissue. PAS stain ; Scale bar = 500 μm
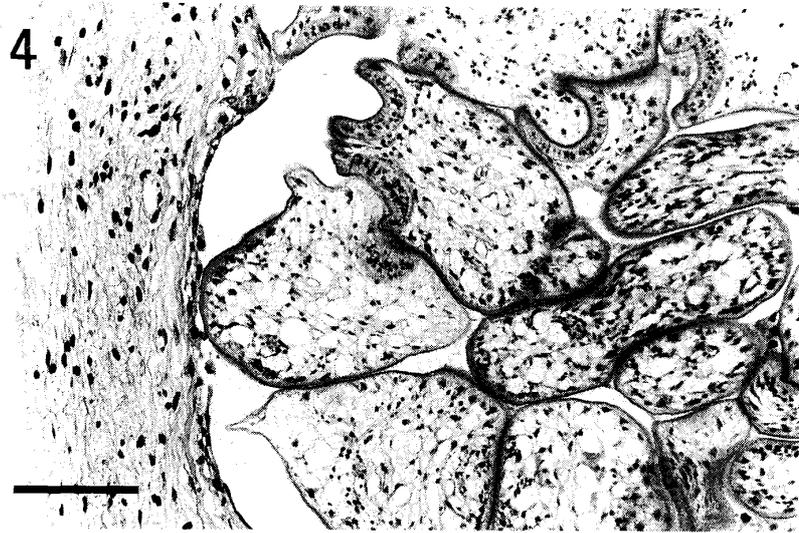
Fig. 4. *E. multilocularis* in subcutaneous tissue of scid mouse at 4 weeks
post-inoculation. Host connective tissue was infiltrated with compara-
tively fewer granulocytes as compared with those of the control
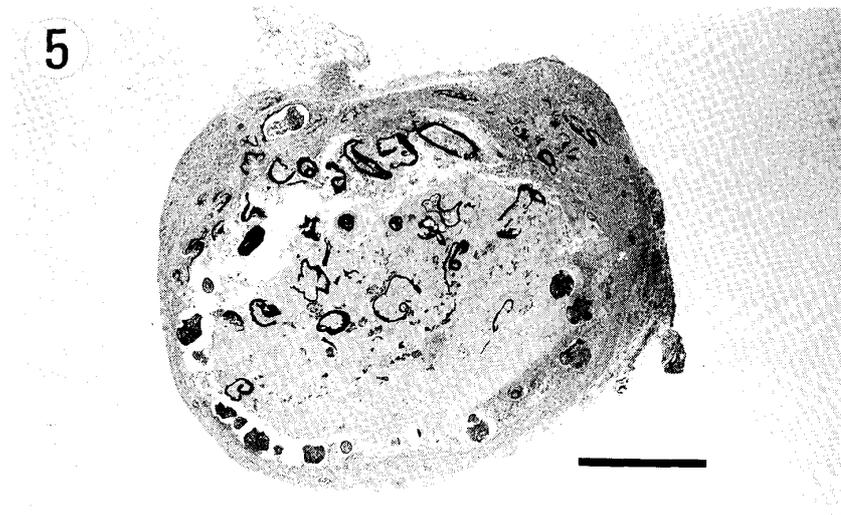C.B-17 mice. HE stain. Scale bar=50 μm

Fig. 5. *E. multilocularis* in subcutaneous tissue of immuno-competent C.B-17
mouse at 4 weeks post-inoculation. Few protoscoleces could be seen
in the lesion surrounded by thick host connective tissue with many
granulocytes. The central necrotic mass contained degenerated worms,
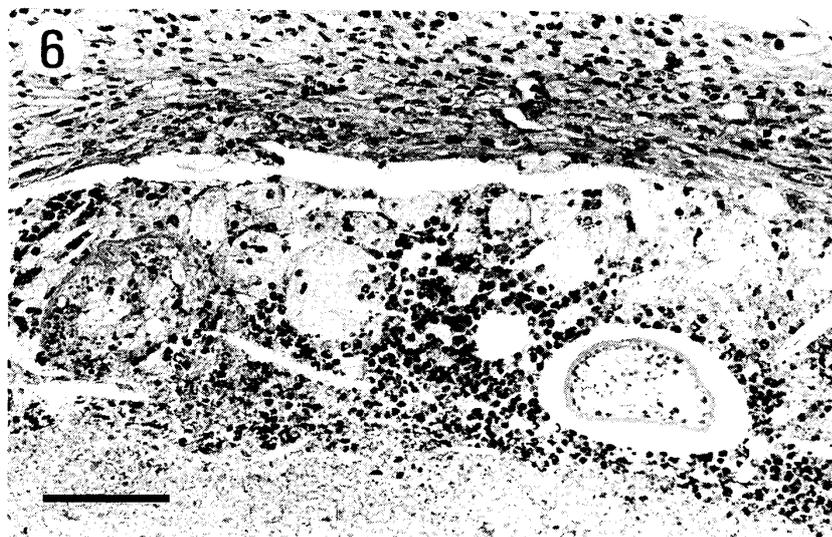neutrophils and laminated layer. PAS stain. Scale bar=500 μm

Fig. 6. *E. multilocularis* in subcutaneous tissue of immuno-competent C.B-17
mouse at 4 weeks post-inoculation. Host connective tissue was infil-
trated with many granulocytes and the worm was surrounded by de-
generated neutrophils. HE stain. Scale bar=50 μm

infection proceeded, the number of hard cyst increased while that of the soft cyst
decreased. The hard cyst was morphologically similar to the secondary hydatid cyst
mass as seen in immune-competent animals, and the soft cyst consisted of fibro-
granulomatous tissue with intact worms.

Worms recovered from all scid mice at 7, 9 and 12 weeks PI showed strobilation
(Fig. 7). More than 30% of worms recovered at 7 weeks PI had one or more
proglottids. As the course of infection proceeded, worms with more proglottids were
recovered (Fig. 8). However, some of the worms showed surface vacuolation and
vesicularization at 9 and 12 weeks PI. The number of these vacuolated worms
increased during the course of infection. Elongation of the genital primordia was
observed in worms recovered in the duration of the experiment (Fig. 9).

Of the 1500 worms transplanted into small intestine of each of the 3 hamsters,
29, 40 and 87 worms (average 52) were recovered from the individual hamster at day
8 post-transplantation. Most of these recovered worms had developed testis, cirrus
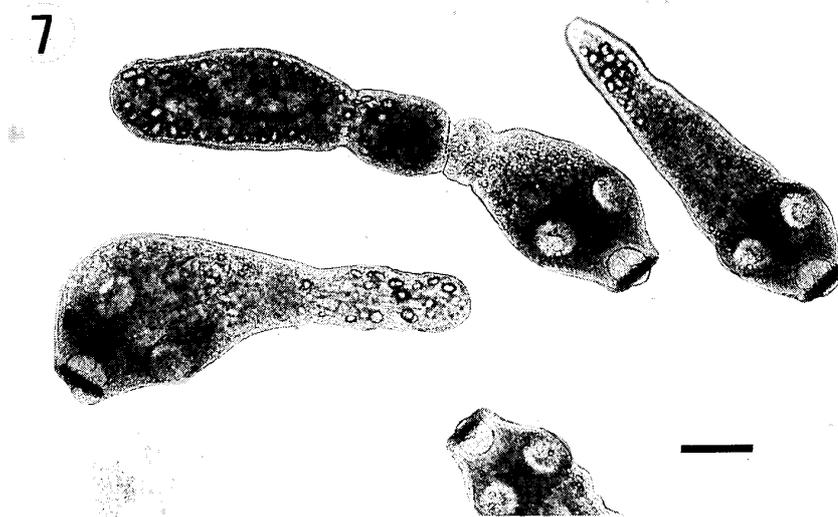sac and conspicuous genital pore (Fig. 10).

Fig. 7. *E. multilocularis* from peritoneal cavity of scid mouse at 7 weeks
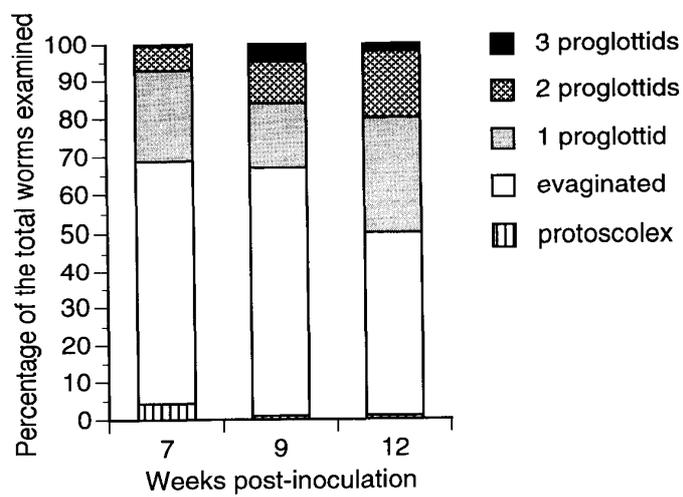post-inoculation. Many worms with proglottids. Scale bar = 100 μm

Fig. 8. Ratio of *E. multilocularis* worms at different strobilation stages re-
covered from peritoneal cavity of scid mice. A total of 100 worms was
examined.

Fig. 9. *E. multilocularis* from peritoneal cavity of scid mouse at 7 weeks post-inoculation. Note the elongation of the genital primordia. Aceto-carmine stain. Scale bar = 100 μm

Fig. 10. *E. multilocularis* recovered from the small intestine of golden hamster at 8 days post-transplantation. Note development of genital organs. Acetocarmine stain. Scale bar = 100 μm

DISCUSSION

Protoscoleces of both *E. granulosus* and *E. multilocularis* are known to be able to develop and grow sexually or asexually depending on their environment[6]. Protoscoleces of *E. multilocularis* that were intrapreritoneally inoculated into the intermediate host such as the mouse usually do not develop to the adult stage but showed asexual proliferation[10,17]. Majority of these inoculated protoscoleces were rapidly surrounded by host granulocytes, macrophages and fibroblasts and finally encapsulated with connective tissue[16]. Such histopathological pictures were seen in the subcutaneous and peritoneal cysts of C.B-17 mice in our experiments.

In a review by Playford *et al.* (1993)[14], humoral and cellular immune components such as granulocytes, eosinophils and macrophages had been implicated in the killing of protoscoleces. Intraperitoneally inoculated protoscoleces of *E. multilocularis* in scid mice had been reported to be intact at 5 weeks PI but at 7 weeks PI, they were surrounded by brood capsule containing newly developed protoscoleces, whereas in the C.B-17 mice, all the protoscoleces of *E. multilocularis* were destroyed at 7 weeks PI[13]. In a further reconsitution experiment using scid mice, suppression of the development of cyst in the lymphocyte-reconstituted scid mice was reported[12]. Another observation on *in vitro* co-culture revealed that the mitogenic effect of protoscoleces of *E. multilocularis* on splenic T and B cells of BALB/c mice depends on IL-1 secreted by cells of macrophage/ monocyte lineage[9]. These observations suggested that not only macrophage and granulocytes but also lymphocyte may play a role in the host protoscolicidal activity.

Results of our first experiment showed that protoscoleces do develop differently at the different inoculation site even in the same host. We also showed in our second experiment that worms recovered from the peritoneal cavity of scid mice can continue to develop to maturity when transplanted in the small intestine of prednisolone-treated golden hamster which served as an alternative definitive host. Our postulation that functional T and/or B lymphocyte might be involved in the immunity against *E. multilocularis* protoscoleces is supported by the following observations ; 1) evaginated protoscoleces from scid mice showed higher viability than those obtained from C.B-17 mice, except those from the cerebrum where there is a blood-brain barrier, 2) body length of worm from scid mice were much longer than those from C.B-17 mice, 3) strobilation coupled with elongation of the genital primordia were seen in worms from scid mice, and 4) host tissue reaction were less severe in scid mice than in C.B-17 mice.

Our histologic findings of the cyst complex in scid mice were the same as described by Playford *et al.* (1992)[13] and the lesser tissue reaction might be conductive for the parenterally inoculated protoscoleces to grow somatically to the stage of the development of genital primordia. Although differentination of sexual organ of the

worm was limited in the scid mice, this limitation could be abolished by transplanting the worm into an alternative definitive host.

The higher viability of worms from the cerebrum than those from other parenteral inoculation sites may be attributed to the absence of encapsulation of the worms by connective tissue in the cerebrum. Vacuolation and vesicularization of the worm have been suggested to be an initial step in the transformation of the protoscolex towards cyst formation[1]. Such phenomenon is also seen in some of the worms recovered from the scid mice in our experiment. Thus, our results suggest that T and/or B lymphocytes may also play a role in determining the fate of transformation of protoscoleces of *E. multilocularis*, that is, whether to develop in the direction of strobilation or cyst formation, at a parenteral site.

ACKNOWLEDGEMENTS

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