Journal of Helminthology (2004) 78(1):1-5.

Hymenolepis microstoma : direct life cycle in immunodeficient mice.

Andreassen J, Ito A, Ito M, Nakao M, Nakaya K.

Journal of Helminthology (2004) 78, 1–5 DOI: 10.1079/JOH2003207

Hymenolepis microstoma: direct life cycle in immunodeficient mice

J. Andreassen[1], A. Ito[2]*, M. Ito[3], M. Nakao[2] and K. Nakaya[4]

[1]Parasitological Laboratory, Department of Population Ecology, University of Copenhagen, Universitetsparken 15, DK-2100 Copenhagen, Denmark: [2]Department of Parasitology and [4]Animal Laboratory for Medical Research, Asahikawa Medical College, Midorigaoka-Higashi 2-1-1-1, Asahikawa 078-8510, Japan: [3]Central Institute for Experimental Animals, Miyamae, Kawasaki 216-0001, Japan

Abstract

The mouse bile duct tapeworm *Hymenolepis microstoma* requires beetles as the obligatory intermediate host. However, when congenitally athymic NMRI-*nu* mice were infected with the mature tapeworm and allowed to eat their own faeces with tapeworm eggs, the oncospheres penetrated the intestinal tissue and developed to cysticercoids. After excysting, growth to adult worms occurs in the lumen of the small intestine and bile duct. Furthermore, the same happened when NMRI-*nu* mice, non-obese diabetic severe combined immunodeficiency (NOD/Shi-*scid*) mice and NOD/Shi-*scid*, IL-2 Rγ^{null} (NOG) mice were orally inoculated with shell-free eggs of this parasite. Differences between the cysticercoids of *H. microstoma* and *H. nana* developed in the mouse intestinal tissues were: (i) the time course for the development of fully matured cysticercoids of *H. microstoma* in mice was about 11 days but only 4 days for *H. nana*; and (ii) cysticercoids of *H. microstoma* developed in mice had a tail while those of *H. nana* had none.

Introduction

The life cycle of *Hymenolepis nana* is of special biological interest, because it represents a modification of the typical indirect two-host tapeworm life cycle, in that the parasite is able to complete its development from egg to adult tapeworm directly in the mammalian host.

We report here that the oncospheres of another hymenolepidid tapeworm, *H. microstoma*, the bile duct tapeworm of mice, which had been thought to require beetles as obligatory intermediate hosts, are able to develop into cysticercoids in the intestinal tissue of congenitally athymic nude mice of an outbred NMRI strain (NMRI-*nu*) available in Denmark, and in non-obese diabetic, severe combined immunodeficient (NOD/Shi-*scid*) and NOD/Shi-*scid*, IL-2 Rγ^{null} (NOG) mice available in Japan (Ito *et al.*, 2002; Yahata *et al.*, 2002). In addition, the time course for maturation of cysticercoids in these mice is much longer than that of *H. nana* in mice and even longer than that of *H. microstoma* in beetles kept at 30°C. The cysticercoids of *H. microstoma* developed in these mice have tails, as do those developed in beetles, while those of *H. nana* developed in mice are devoid of tails.

*Author for correspondence
Fax: +81-166-68-2429
E-mail: akiraito@asahikawa-med.ac.jp

Materials and methods

Experimental animals

All mice for experimental infections in Japan were specific pathogen free individuals purchased from CLEA-Japan in Tokyo (BALB/cA mice, BALB/cA-*nu* mice, C.B-17-*scid* mice of both sexes between 6 and 10 weeks old) and Central Institute for Experimental Animals in Kawasaki, Japan (NOD/Shi-*scid* and NOD/Shi-*scid*, IL-2 Rγ^{null} mice (abbreviated as NOD-*scid* and NOG mice,

respectively, Ito *et al.*, 2002) of both sexes between 6 and 10 weeks old). NMRI-*nu* mice of both sexes between 1 and 7 months old were reared from the breeding of NMRI-*nu*/+ females and NMRI-*nu* males, purchased from Bomholtgaard Co., Denmark, under conventional conditions. All experiments using these mice were permitted by the animal ethics committees in Denmark and Asahikawa Medical College. Five male or female mice were kept together in each cage and all cages were changed once every week. All NOD-*scid* and NOG mice were reared at the Central Institute for Experimental Animals and maintained at the Animal Laboratory at Asahikawa Medical College in isolated boxes (CLEA, Japan: EBAC-L) to prevent any microbial infections throughout the experiment. All cages were autoclaved along with the wood chip bedding and drinking water. Cobalt 60 (50 KGY) irradiated sterilized pellet food (CLEA, Japan; CL-2) and sterilized drinking water were taken *ad libitum*. Food and water for NMRI-*nu* mice were not Cobalt 60 irradiated or autoclaved. Specific pathogen free, immunocompetent BALB/cA mice purchased from CLEA, Japan, used for experimental infections and for preparation of eggs from mature adult worms, were kept in conventional animal rooms.

Experimental infections

Experimental infections with either five cysticercoids or approximately 5×10^3 eggs suspended in 0.2 ml PBS were carried out using stomach tubes whilst under light CO_2/O_2 anaesthesia in Copenhagen, whereas infections with either five cysticercoids or 2×10^4 or 6×10^4 shell-free eggs suspended in about 0.2 ml sterile PBS were carried out using sterilized stomach tubes whilst under light ether anaesthesia in Asahikawa. NMRI-*nu* mice given five cysticercoids of *H. microstoma* were killed at different intervals between 48 and 139 days post infection (p.i.). The liver and small intestine were removed and placed in a black wax-tray. The small intestine was stretched out, fixed by pins at the pylorus and the caecum and opened lengthwise. Adult worms were removed and counted. The intestine was then divided into ten equal sections, pressed in a trichine compressorium and cysticercoids counted under the microscope. Mice orally inoculated with different doses of eggs were killed from 2 to 34 days p.i. During the experiments, uninfected control mice were kept in a similar way in the same animal room, and none of these mice were infected with any stage of the tapeworms. Ito *et al.* (1989) showed that the mice were coprophagous, and this was verified again during these experiments. Furthermore, NMRI/*nu* mice have been infected with *H. diminuta* for many years in our laboratory, and, although we have observed coprophagy in these mice, naïve mice have not been infected with any tapeworm and infected mice never harboured more worms than the number of cysticercoids administered.

Parasite material

Hymenolepis microstoma, originally from the Wellcome Laboratories for Experimental Parasitology, Glasgow University, has been maintained in our laboratories using mice and flour beetles (*Tribolium confusum*) since 1970 in Denmark and since 1985 in Japan. Eggs of *H. microstoma* were teased from gravid proglottids of adult worms recovered from BALB/cA mice from one to 12 months after oral inoculation with five cysticercoids each. About 200 BALB/cA mice inoculated with five cysticercoids each were used for preparation of eggs for experimental infections in Japan. All egg suspensions were rinsed several times with sterile PBS and the shells of more than 50% of eggs removed by cracking them with glass beads just before experimental inoculation in order to increase the infection rates as in *H. nana* (Berntzen & Voge, 1965). Cysticercoids of *H. microstoma* were prepared from beetles which were fed with gravid segments for 1–3 months and kept at 25°C. All cysticercoids were prepared from beetles just a few hours before experimental infection and rinsed with sterile PBS several times at room temperature.

Results

Hymenolepis microstoma *in NMRI*-nu, *BALB/cA*-nu *and C.B-17*-scid *mice*

About 200 BALB/cA mice were inoculated with five cysticercoids each and killed 1–12 months later in order to prepare eggs. No mouse harboured more than five adult worms. In immunocompetent NMRI- + /nu and NMRI- + /+ mice the number of tapeworms established was never more than the number cysticercoids administered. However, when two immunodeficient NMRI-*nu* mice were inoculated with five cysticercoids each and killed 4 months later, they harboured 19 and 91 small adult worms with various sizes from 2–10 mm long, plus five large mature worms. Additional NMRI-*nu* mice in Denmark and BALB/cA-*nu* and C.B-17-*scid* mice in Japan were inoculated with five cysticercoids in order to confirm this unexpected finding. Of 22 NMRI-*nu* mice inoculated with five cysticercoids of *H. microstoma*, 13 mice were found to harbour either more than five worms and/or cysticercoids in the intestinal tissue (table 1) when they were killed between 48 and 139 days p.i. The number of cysticercoids and adult worms per mouse was highly variable (table 1). The highest number of adult worms in the bile duct and small intestine was 116 in a mouse killed 132 days p.i. The overall morphology of the cysticercoid (fig. 1), which is similar to that of cysticercoids developed in beetles, consists of a retracted rostellum with four suckers and a crown of small hooks at the anterior end, and an elongated tail. The distribution of cysticercoids in the intestine of 12 NMRI-*nu* mice is summarized in fig. 2. The maximum number of cysticercoids in the intestinal villi was 589 found in a mouse autopsied 101 days p.i. More than 80% of the cysticercoids were located in the intestinal tissue between 10 and 30% of the distance from the pylorus of NMRI-*nu* mice (fig. 2).

In contrast, none of the 21 BALB/cA-*nu* or 30 C.B-17-*scid* mice or 31 immunocompetent BALB/cA mice harboured any cysticercoids in the small intestine nor any adult worms of the second generation following infection with five *H. microstoma* for up to five months.

Table 1. The number of adult worms and cysticercoids recovered from 15 NMRI-*nu* mice killed between 48 and 139 days post-inoculation (p.i.) with five cysticercoids of *Hymenolepis microstoma*.

Days p.i.	No. of adult worms	No. of cysticercoids
48	4	8
48	5	5
58	60	166
72	23	0
73	35	93
97	9	8
101	44	589
114	19	NE
114	91	NE
115	95	193
115	6	3
122	7	13
132	116	88
133	10	26
139	21	15

NE, not examined.

*Development of cysticercoids in NMRI-*nu *and C.B-17-*scid *mice from eggs inoculated orally*

In a preliminary attempt to infect naïve nude mice directly with eggs, two NMRI-*nu* mice were given 5×10^3 shell-free eggs of *H. microstoma* orally and killed 7 days p.i. and 14 days p.i. No cysticercoids or adult worms were found in the mouse killed 7 days p.i. but one immature adult worm (about 4 mm long) was found in the first 10% section of the small intestine and two mature cysticercoids were found (one from 0–10% and one from 10–20%) in the intestinal tissue 14 days p.i. In contrast, when 20 C.B-17-*scid* mice, divided into two groups of ten each and orally given 5×10^3 or 2×10^4 shell-free eggs, were killed 14 or 21 days p.i., neither any cysticercoids in the intestinal tissue nor adult worms in the intestinal lumen were found.

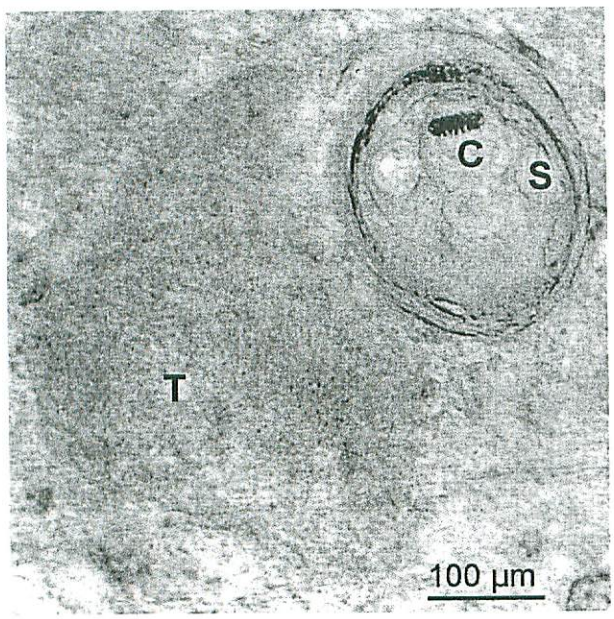

Fig. 1. A cysticercoid of *Hymenolepis microstoma* developing in the small intestine of an NMRI-*nu* mouse. The small intestine was pressed between two glass plates in a trichine compressorium. C, crown of small hooks on the retracted rostellum; S, one of four suckers in the scolex, three are visible; T, elongated tail of the cysticercoid.

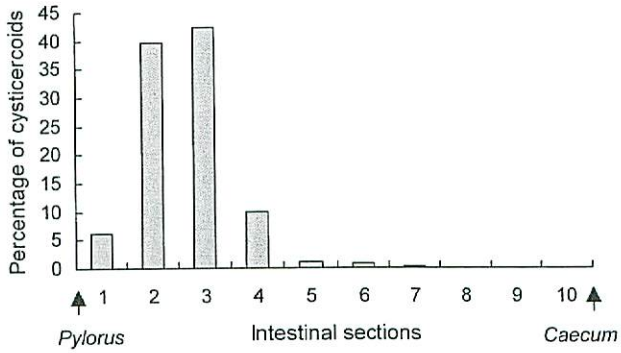

Fig. 2. The distribution of *Hymenolepis microstoma* cysticercoids in the small intestine of NMRI-*nu* mice autopsied 48 to 139 days post-infection with five worms. The ten intestinal sections from pylorus to caecum are equal in length.

The direct life cycle of H. microstoma *in NOD-*scid *and NOG mice*

When two female NOD-*scid* mice were infected with five cysticercoids and killed 60 days p.i., one harboured five large adult worms in the intestinal lumen and one cysticercoid in the intestinal tissue. The other female mouse harboured large worms and one immature worm (about 2 mm long) in the intestinal lumen and two cysticercoid capsules without cysticercoid larvae in the intestine (fig. 3a). The presence of cyst capsules in the upper part of the intestine was confirmed by the detection of oncospheral paired hooks in the tail of the cysts. Fifteen NOD-*scid* mice of both sexes were orally given about 2×10^4 shell-free eggs and five mice were killed 4, 10 and 11 days p.i. respectively. Although no fully developed cysticercoids were found by 4 days p.i., a single cysticercoid was recovered from two of five mice 10 days p.i. and a single cysticercoid from one mouse and two cysticercoids from another of the five mice killed 11 days p.i. The two cysticercoids grown in NOD-*scid* mice by 10 days p.i. had a fully developed withdrawn scolex (fig. 1), whereas the three cysticercoids developed by 11 days p.i. were actively moving for excystment in the cyst capsules, similar to *H. nana* (see figs 8–20 in Ito *et al.*, 1977). Further evidence was obtained from NOD-*scid* mice inoculated with 6×10^4 eggs and killed 12 days p.i. and 34 days p.i. From one of two NOD-*scid* mice killed 12 days p.i., a juvenile worm (2 mm long) was found. Two adult worms were recovered from each of two of four NOD-*scid* mice killed 34 days p.i. One mouse harboured one large worm of 97.0 mg biomass (wet worm weight just after removal of surface PBS on a filter paper) and a small one of 5.0 mg in the bile duct, whereas the other mouse harboured one large worm of 59.0 mg and a small

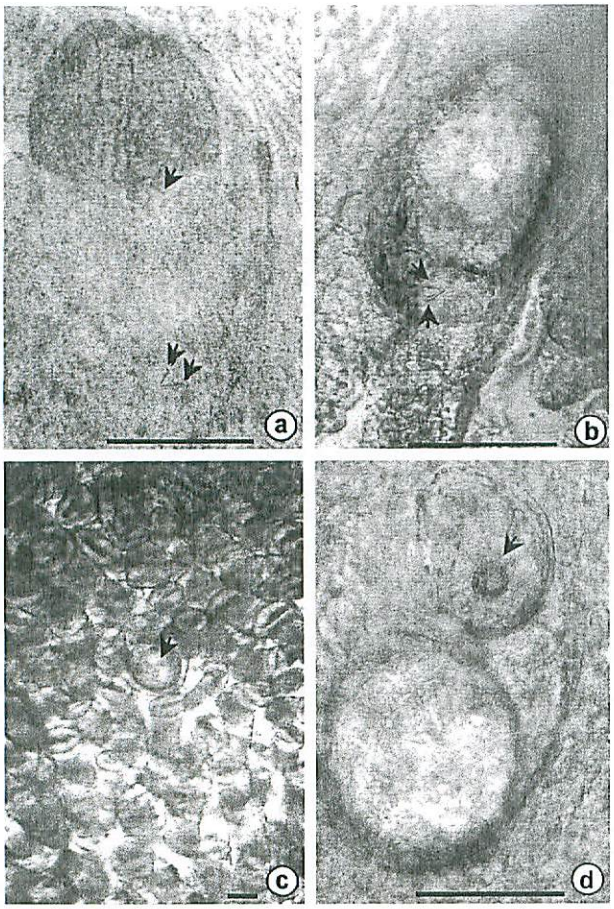

Fig. 3. (a) Cyst capsule without the cysticercoid of *Hymenolepis microstoma* 12 days post-infection (p.i.) in a NOD-*scid* mouse (arrowheads: two pairs of oncospheral hooks in the tail). (b) Immature cysticercoid 8 days p.i. in a NOG mouse (arrowheads: oncospheral hooks). (c) Cysticercoid (arrowhead) in a villus of the small intestine. (d) Mature cysticercoid 10 days p.i. in a NOG mouse (arrowhead: rostellum hooks in the evaginated cysticercoid. Scale bar = 100 μm.

worm of 1.7 mg in the upper part of the small intestine. There was no evidence of parasite invasion into the bile duct in the latter mouse.

Additional work was carried out using NOG mice inoculated with 6×10^4 eggs. Two mice were killed every two days after egg inoculation. On days 2 and 4 p.i. it was impossible to detect any evidence of cysticercoid development in the intestinal tissue under the microscope. However, on day 6 p.i. two immature cysticercoids of about 475 μm length were detected from one of the two mice. Oncospheral hooks of 17.5 μm were observed. On day 8 p.i. one immature cysticercoid was detected from one of two mice (fig. 3b). On day 10 p.i., four mature cysticercoids were detected from one mouse and three from the other mouse (fig. 3c,d).

Discussion

The present discovery that orally inoculated or eaten eggs of *H. microstoma* may develop into cysticercoids in the tissue of the small intestine and further excyst and develop into adult worms in the bile duct and/or in the lumen of the small intestine of immunodeficient, NMRI-*nu*, NOD-*scid* and NOG mice was unexpected. However, this could have been predicted because it has been shown that specific antibodies to oncospheres are produced in immunocompetent mice either orally inoculated with viable eggs or through coprophagy of faeces from mice harbouring mature adult worms of *H. microstoma* (Ito *et al.*, 1989). Furthermore, when shell-free eggs were inoculated orally into naïve mice, oncospheres were able to hatch and invade the tissue in the anterior third of the small intestine, but these oncospheres did not develop into cysticercoids (Onitake *et al.*, 1990). The present finding that more than 80% of cysticercoids were located in the intestinal tissue between 10 and 30% of the distance from the pylorus of NMRI-*nu* mice (fig. 2) is in accordance with the finding of oncospheres only in the lamina propria of the posterior half of the first third of the small intestine in normal immunocompetent mice (Onitake *et al.*, 1990).

The morphology of cysticercoids of *H. microstoma* which developed in the intestinal tissue of NMRI-*nu*, NOD-*scid* and NOG mice appears to be similar to that of cysticercoids in the intermediate host *Tribolium confusum* (Voge, 1964) or those developed after *in vitro* cultivation (Seidel, 1975). This is different from the cysticercoid of *H. nana* which has a tail in beetles but is tailless in mice (table 2) (Voge & Heyneman, 1957; Ito *et al.*, 1977).

The time course of development of cysticercoids of *H. microstoma* in the intestinal tissue after inoculation with shell-free eggs (about 11 days) shows that the development of *H. microstoma* from oncosphere to cysticercoid in the intestinal tissue is much longer than the 4 days taken for *H. nana*, and longer than its development in the intermediate host *T. confusum*, which takes 7–8 days at

Table 2. Differences in the time of development and morphology of the cysticercoids of *Hymenolepis microstoma* and *H. nana* in mice.

Species	Host	Cysticercoid morphology	Days for development
H. microstoma	NMRI-*nu* mice	Tailed	<14
	NOD/Shi-*scid* mice	Tailed	11
	NOD/Shi-*scid*, IL-2 Rγ ^{null} mice	Tailed	10–11
	Beetle	Tailed	7–8 at 30°C[a]
H. nana	Beetle	Tailed	7–8 at 30°C[a]
	Mice	Tailless	4[a,b]

[a] Voge & Heyneman (1957).
[b] Ito *et al.* (1977).

30°C (table 2). The discovery of a direct cycle of *H. microstoma* in NMRI-*nu*, NOD-*scid* and NOG mice raises the important question as to why *H. microstoma* has a direct life cycle in these immunodeficient mice, but not in other immunodeficient mice such as BALB/cA-*nu* or C.B-17-*scid* mice or immunocompetent mice. The lack of macrophages and natural killer (NK) cell activity in NOD-*scid* and NOG compared with C.B-17-*scid* mice (Christianson *et al.*, 1997; McGuirk *et al.*, 1998; Robin *et al.*, 1999; Ito *et al.*, 2002; Yahata *et al.*, 2002), is likely to be responsible for the development of oncospheres of *H. microstoma* to cysticercoids in these mice.

Schmidt (1986) argued that *H. nana* and *H. microstoma* should be differentiated from *H. diminuta* and put in a separate genus. This may be supported by the evidence of cross immunity between *H. microstoma* and *H. nana* (Ito *et al.*, 1989), and from nuclear ribosomal and mitochondrial DNA sequences of the three species (Okamoto *et al.*, 1997). Furthermore, the discovery of a direct life cycle of *H. microstoma* in NMRI-*nu*, NOD-*scid* and NOG mice lends support to the idea that *H. microstoma* is phylogenetically more closely related to *H. nana* than *H. diminuta*, which has never developed cysticercoids in the immunodeficient NMRI-*nu* and other strains of nude mice in our laboratories.

Acknowledgements

We thank Sylvia Mathiasen for careful technical assistance, Dr J. Lützen for photographical help and David Nash for English correction in Copenhagen and Dr Marcello O. Sato for preparation of figures in Asahikawa. A. Ito and M. Ito thank the Japan Society of Promotion of Science for a grant-in-aid for International Scientific Research (Joint Research (A) 14256001) to AI, and for Scientific Research (B) (13558100) to MI.

We (JA and AI) dedicate this unexpected finding of the direct life cycle of *Hymenolepis microstoma* in immunodeficient mice to the late Emeritus Professor James Desmond Smyth, who died on 4th January 1999. He encouraged us to do research on the biology of cestodes.

References

Berntzen, A.K. & Voge, M. (1965) *In vitro* hatching of oncospheres of four hymenolepidid cestodes. *Journal of Parasitology* **51**, 235–242.

Christianson, S.W., Greiner, D.L., Hesselton, R.A., Leif, J.H., Wagar, E.J., Schweitzer, I.B., Rajan, T.V., Gott, B., Roopensian, D.C. & Schultz, L.D. (1997) Enhanced human CD4+ T cell engraftment in beta2-microglobulin-deficient NOD-scid mice. *Journal of Immunology* **158**, 3578–3586.

Ito, A., Yamamoto, M. & Okamoto, K. (1977) Photomicroscopic observation of infective cysticercoid and the earlier stages of *Hymenolepis nana* isolated from the mouse intestine. *Japanese Journal of Parasitology* **26**, 345–349.

Ito, A., Itoh, M., Andreassen, J. & Onitake, K. (1989) Stage-specific antigens of *Hymenolepis microstoma* recognized in BALB/c mice. *Parasite Immunology* **11**, 453–462.

Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., Ueyama, Y., Koyanagi, Y., Suganuma, K., Tsuji, K., Heike, T. & Nakahata, T. (2002) NOD/SCID/γ_c^{null} mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* **100**, 3175–3182.

McGuirk, J., Yan, Y., Childs, B., Fernandez, J., Barnett, L., Jagiello, C., Collins, N. & O'Reilly, R.J. (1998) Differential growth patterns in SCID mice of patient-derived chronic myelogenous leukemias. *Bone Marrow Transplantation* **22**, 367–374.

Okamoto, M., Agatsuma, T., Kurosawa, T. & Ito, A. (1997) Phylogenetic relationships of three hymenolepidid species inferred from nuclear ribosomal and mitochondrial DNA sequences. *Parasitology* **115**, 661–666.

Onitake, K., Sasaki, J., Andreassen, J. & Ito, A. (1990) *Hymenolepis microstoma*: oncospheres invade the intestinal tissue of the definitive host. *Journal of Helminthology* **64**, 168–170.

Robin, C., Pflumio, F., Vainchenker, W. & Coulobmbel, L. (1999) Identification of lymphomyeloid primitive progenitor cells in fresh human cord blood and in the marrow of nonobese diabetic severe combined immunodeficient (NOD-SCID) mice transplanted with human CD34 (+) cord blood cells. *Journal of Experimental Medicine* **189**, 1601–1610.

Schmidt, G.D. (1986) *CRC handbook of tapeworm identification*. 675 pp. Boca Raton, CRC Press.

Seidel, J.S. (1975) The life cycle *in vitro* of *Hymenolepis microstoma* (Cestoda). *Journal of Parasitology* **61**, 677–681.

Voge, M. (1964) Development of *Hymenolepis microstoma* (Cestoda: Cyclophyllidea) in the intermediate host *Tribolium confusum*. *Journal of Parasitology* **50**, 77–80.

Voge, M. & Heyneman, D. (1957) Development of *Hymenolepis nana* and *Hymenolepis diminuta* (Cestoda: Hymenolepididae) in the intermediate host *Tribolium confusum*. *University of California Publications in Zoology* **59**, 549–579.

Yahata, T., Ando, K., Nakamura, Y., Ueyama, Y., Shimamura, K., Tamaoki, N., Kato, S. & Hotta, T. (2002) Functional human T lymphocyte development from cord blood CD34$^+$ cells in nonobese diabetic/Shi-scid, IL-2 receptor γ null mice. *Journal of Immunology* **169**, 204–209.

(Accepted 12 August 2003)