In Vitro Cultivation of the Third and Fourth Stage Larvae of *Angiostrongylus cantonensis*

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(Received 16 July 1992/Accepted 6 January 1993)

ABSTRACT. The third and fourth stage larvae of *Angiostrongylus cantonensis* were cultured in various media. When the third stage larvae were cultured in RPMI 1640 supplemented with 20% fetal calf serum for four weeks, about 30% developed to the late third stage. On the other hand, when the fourth stage larvae recovered from rat brains were cultured in Waymouth's chemically defined medium (MB 752/1) for one week, the worms grew rapidly and 74% developed into young adults. The mean body length of the worms in Waymouth's medium showed a 1.4-fold increase in size compared with that before culture.—KEY WORDS: *Angiostrongylus cantonensis*, chemically defined medium, *in vitro* culture.

J. Vet. Med. Sci. 55(2): 345–347, 1993

Although *Angiostrongylus cantonensis* causing cerebrospinal angiostrongylosis is known as an important human parasitic nematode, only a few reports have been published concerning the *in vitro* cultivation of parasites that belong to genus *Angiostrongylus* [8, 10, 11, 13]. Recently, we successfully cultured the intramolluscan stage of *A. cantonensis* from the first stage to the infective third stage larvae which showed relatively uniform development similar to those *in vivo* [4]. However, concerning the cultivation of *A. cantonensis* from the third stage, there have been no reports on successful culture. The present paper describes an attempt to culture *A. cantonensis* from the third or fourth stage larvae to young adults.

The third stage larvae of *A. cantonensis* used for cultures were collected by pepsin digestion and a Baermann apparatus from infected *Biomphalaria glabrata* (Puerto Rican strain) [5]. Fourth stage larvae of *A. cantonensis* used for cultures were recovered with a Baermann apparatus from rat brains. These isolated larvae were washed with Earle's balanced salt solution (EBSS) containing antibiotics. Then, 50 to 100 third stage larvae or 10 to 20 fourth stage larvae were cultured in a tissue culture flask (25 cm^2, Corning) containing 10 m*l* of culture medium at 37°C under 5% CO_2 in air. The medium was changed once a week.

Evaluation of the cultures was mostly based on the following criteria: (a) Early third stage larvae (early L3): before culture; Late third stage larvae (late L3): increase in body size and enclosed in the sheath of the third molt, chitinous rod-shaped structures visible in the vestibule. (b) Fourth stage larvae (L4): differentiable gender; increase in length and enclosed within the sheath of the fourth molt, with the chitinous rod-shaped structures having disappeared. (c) Young adult worms: the sheath of the fourth molt cast off and increase in length and formation of the bursa and vulva.

Culture media were prepared as follows: Eagle's Minimum Essential Medium (MEM) (GIBCO), NCTC 135 (GIBCO), Waymouth's medium MB 752/1 (WYM) (GIBCO), TC 199 (GIBCO), RPMI 1640 (GIBCO) and Earle's BSS (EBSS) (Nissui) were obtained from commercial sources. Fetal calf serum (FCS), rat red blood cells (RBCs) and tryptose phosphate broth (TPB) (Difco) were prepared according to the method of Hata and Kojima [5]. All culture media contained penicillin and streptomycin at concentrations of 100 units ml^{-1} and 50 μg ml^{-1}, respectively.

Table 1 shows the development of the third stage larvae of *A. cantonensis* in various media. When the larvae were cultured in various chemically defined media without serum supplement for 4 weeks, 9% of the worms in RPMI 1640 and 4% of the worms in NCTC 135 developed to the late third stage. However, none of the worms developed to the late third stage in WYM and Eagle's MEM. On the other hand, when these defined media were supplemented with 20% FCS, larval development was observed in all media. Especially, in RPMI 1640 supplemented with 20%

Table 1. Development of third stage larvae of *Angiostrongylus cantonensis* in various media at 4 weeks after cultivation

Medium	Percentage development		Survival rate (%)
	Early L3	Late L3	
WYM	100	0	6
Eagle's MEM	100	0	6
NCTC 135	96	4	16
RPMI 1640	91	9	57
WYM+20% FCS	93	7	49
Eagle's MEM+20% FCS	80	20	87
NCTC 135+20% FCS	75	25	84
RPMI 1640+20% FCS	69	31	57
RPMI 1640+20% FCS+RBCs	81	19	77
RPMI 1640+20% FCS+10% TPB	79	21	71

FCS, more than 30% of the worms developed to the late third stage, although these worms gradually died thereafter. The additions of rat RBCs and 10% TPB to RPMI 1640 containing 20% FCS did not provide any additional benefit for worm development.

Fourth stage larvae of *A. cantonensis* recovered from rat brains on day 7 post-inoculation with third stage larvae were cultured in various media for 7 days. As shown in Table 2, when the fourth stage larvae were cultured in WYM or NCTC 135, they developed into young adults. The most suitable medium for the development was chemically defined WYM, as 74% of the worms developed to young adults in this medium. Mean body length of the worms cultured in WYM was 1.76 mm ± 0.39 (n=40), which showed about 1.4-fold increase in size compared with that of before culture. However, when the cultivation was continued, these young adult worms gradually died. None of the worms developed to young adult in RPMI 1640, the best developmental environment observed for the third stage larvae. However, when RPMI 1640 was supplemented with 20% FCS, the larvae developed to young adults. The addition of FCS to WYM did not have any additional enhancing effect on worm development. Further, the additions of rat RBCs and 10% TPB to WYM supplemented with 20% FCS also did not lead to any increased larval development.

Although some parasitic nematodes of vertebrates have been successfully cultured to maturity [1, 2, 9], there have been no reports on worms successfully cultured to adult worms in chemically defined medium. Recently, however, we successfully cultured *A. costaricensis* from the third stage to young adults in Waymouth's chemically defined medium [5]. In the present study also, the fourth stage larvae of *A. cantonensis* developed to young adults in chemically defined WYM. Other chemically defined media did not induce development like WYM. Furthermore, Hata *et al.* [6] reported that when filariform larvae of *Necator americanus* were cultured in various media, the best larval development to the fourth stage was achieved in WYM with 20% FCS. Thus, WYM may be useful for the cultivation of other parasites *in vitro*. WYM was designed for the culture of mammalian cells without serum supplementation [12]. This medium is formulated relatively simply, as it contains 18 amino acids, 11 vitamins, 8 inorganic salts, as well as glucose, glutathione, hypoxanthine and phenol red. Among the components, lysine, histidine, choline, thiamine, hypoxanthine and dextrose were prepared at higher concentrations than in the other chemically defined media tested, and perhaps this was responsible for the greater larval development. However, further research will be required to clarify the effects of all the components essential for development, in terms of both their individual activity and their combined effect.

Table 2. Development of fourth stage larvae of *Angiostrongylus cantonensis* recovered from rat brains on day 7 after inoculation with third stage larvae in various media

Medium	Percentage development			Survival rate (%)
	L4	Exsheathing	Young adult	
Earle's BSS	100	0	0	0
WYM	13	13	74	73
NCTC 135	21	43	36	43
TC 199	88	12	0	13
RPMI 1640	87	13	0	22
NCTC 135+20% FCS	24	23	53	82
RPMI 1640+20% FCS	20	39	41	68
WYM+20% FCS	7	20	73	87
WYM+20% FCS+RBCs	27	18	55	43
WYM+20% FCS+10% TPB	33	7	60	80

Observations were made on day 7 after cultivation.

Although the fourth stage larvae of *A. cantonensis* developed well in WYM but not in RPMI 1640, the third stage larvae developed well in RPMI 1640 but not in WYM. Thus, the suitable medium for the development of *A. cantonensis* differed according to the developmental stages of worms. These results indicate that nutritional and/or physiological requirements of *A. cantonensis* may be different in the various developmental stages, reflecting a change in parasitizing locations in the final host.

In the final host, the larvae of *A. cantonensis* casts off the third and fourth sheaths at six to seven days and eleven to thirteen days after infection, respectively [7]. In the present study, however, they took two weeks to reach the late third stage in RPMI 1640 supplemented with 20% FCS. Growth and development of parasitic helminths *in vitro* tend to be slower, and the cultured worms smaller than those obtained *in vivo* [1–3, 8]. However, in the cultivation of fourth stage larvae (recovered from rats on day 7 post-inoculation of third stage larvae) in WYM, they cast off the fourth sheath at three to six days after cultivation (10 to 13 days old after infection of rats). Thus, the growth speed of fourth stage larvae *in vitro* did not decelerate compared with that *in vivo*. Moreover, the size of the young adult worms immediately after exsheathment in WYM was not so different from that of worms obtained from *in vivo* experiments [7].

In the cultivation of *A. costaricensis*, Hata and Kojima [5] reported that the addition of RBCs to WYM was essential for the development of young adult worms, although it was not required for the development up to the young adult stage. In the present study also, the addition of RBCs to the medium did not appear to benefit the development of the worms until the young adult stage, although RBCs were incorporated and filled in their intestines.

ACKNOWLEDGEMENT. This study was partially supported by a Grant-in-Aid for Scientific Research (No. 04670225) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Douvres, F. W. and Urban, J. F. 1987. Nematoda except

parasites of insects. pp. 318–378. *In*: *In Vitro* Methods for Parasite Cultivation (Taylor, A.E.R. and Baker, J. R. eds.), Academic Press, New York.
2. Hansen, E. L. and Hansen, J. W. 1978. Nematoda parasitic in animals and plants. pp. 227–277. *In*: Methods of Cultivating Parasites *in Vitro*, 1st ed. (Taylor, A.E.R. and Baker, J.R. eds.), Academic Press, New York.
3. Hata, H., Yokogawa, M., Kobayashi, M., Niimura, M., and Kojima, S. 1987. *J. Parasitol.* 73: 792–796.
4. Hata, H. and Kojima, S. 1990. *Exp. Parasitol.* 70: 476–482.
5. Hata, H. and Kojima, S. 1991. *Exp. Parasitol.* 73: 354–361.
6. Hata, H., Paz, K. C., Miranda, P. J., and Tateno, T. 1992. *Ann. Trop. Med. Parasitol.* 86: 157–162.
7. Mackerras, M. J. and Sandars, D. F. 1955. *Aust. J. Zool.* 3: 1–25.
8. Moreau, J. P. and Lagrauret, J. 1972. *Ann. Parasitol.* 47: 525–529.
9. Taylor, A. E. R. and Baker, J. R. 1968. The Cultivation of Parasites *in Vitro*. p. 377. Blackwell, Oxford and Edinburgh.
10. Uga, S., Araki, K., and Matsumura, T. 1984. *Jpn. J. Parasitol.* 33: 257–264.
11. Uga, S. and Matsumura, T. 1982. *Jpn. J. Parasitol.* 31: 59–66.
12. Waymouth, C. and Jackson, R. B. 1959. *J. Natl. Cancer Inst.* 22: 1003–1017.
13. Weinstein, P. P., Rosen, L., Laqueur, G. L., and Sawyer, T. K. 1963. *Am. J. Trop. Med. Hyg.* 12: 358–377.