Journal of Helminthology (2001) **75,** 351–354 DOI: 10.1079/JOH200160

Effects of anthelmintics on the development of eggs of *Angiostrongylus costaricensis* in vitro

A. Ishih*, M. Yanoh, C. Ikeya, A. Ban and M. Terada

Department of Parasitology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

Abstract

Effects of the anthelmintics, pyrantel and levamisole, on egg development of *Angiostrongylus costaricensis* were studied *in vitro*. After 7 days, about 80% of eggs developed to first-stage larvae in Ham's F-12 medium with 10% foetal calf serum under 5% CO_2. Significant inhibition of development was caused by pyrantel (10^{-9}–10^{-8} g ml^{-1}) and levamisole (10^{-9}–10^{-8} g ml^{-1}) (Mann-Whitney *U*-test; $P < 0.05$), and none of the eggs developed to first-stage larvae in higher concentrations of these anthelmintics (10^{-7} g ml^{-1}). Furthermore, incubation with these drugs at 10^{-8} g ml^{-1} for at least 3 h or at 10^{-4} g ml^{-1} for 1 h caused irreversible effects on egg development.

Introduction

Following treatment of tissue parasites, adverse effects on the host are often caused by allergic reaction to killed worms. *Angiostrongylus costaricensis* is a tissue nematode which causes human abdominal angiostrongylosis. Effective treatments for this disease have not been identified. Since *A. costaricensis* develops to the adult stage in humans, *in vitro* effects of anthelmintics on the adult stage of this nematode have been widely examined (Terada *et al.*, 1992; Tungtrongchitr *et al.*, 1992). Eggs deposited by adult worms in mesenteric arteries become lodged in terminal arterioles, and thereafter develop and hatch to first-stage larvae in intestinal tissues (Morera, 1973). Eggs and larvae in the small vessels of the tissues induce a strong granulomatous inflammatory reaction, and become surrounded by massive eosinophilic infiltration, especially in the mucosa and submucosa of the intestinal wall (Morera, 1985). Therefore, the study of effects of drugs on the development of eggs to first-stage larvae may provide information useful to the development of new approaches for chemotherapy of abdominal angiostrongylosis characterized by intestinal granuloma. Ishih (1994) suggested that in rats infected with *A. cantonensis*, levamisole affects larval output through a direct paralysing action on the first-stage larvae and an indirect effect on eggs, including inhibition of energy metabolism. In the present study, we investigated the effects of anthelmintics on egg development in *A. costaricensis* in vitro.

*Fax: 81 53 435 2337
E-mail: aishih@hama-med.ac.jp

Materials and methods

Egg recovery

Angiostrongylus costaricensis was maintained in our laboratory by cyclical passage through cotton rats (*Sigmodon hispidus*) and *Biomphalaria glabrata* snails, and all animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine. Infected cotton rats were euthanized by a diethyl ether overdose 2 to 4 months after infection. Adult worms were recovered from the mesenteric artery, transferred into Petri dishes (3.5 cm in diameter) containing Ham's F-12 medium (Hata, 1996) with 100 units of penicillin ml^{-1} and 100 μg of streptomycin ml^{-1} then washed three times with the same medium. Worms were transferred into another dish containing Ham's F-12 medium supplemented with 10% inactivated foetal calf serum (culture medium) and incubated for 2 h at 37°C under 5% CO_2. After incubation, eggs deposited into the medium were collected in a centrifuge tube and washed three times in culture medium by centrifugation at 1000 rpm for 1 min. The eggs were suspended in 2 ml of the culture medium for experimentation.

Culture conditions

As the depth of the culture medium can influence the rate of oxygen diffusion into the cells (Freshney, 1987), the effects of culture medium volume on egg development was determined. Assays were carried out using 96-well plates (Corning). About 20 eggs per well were transferred to yield a final volume of 100 μl or 200 μl. The plates were kept at 37°C under 5% CO_2 for 1 week. The culture medium was not changed during the incubation. Developmental stages were classified as cleavage stage, tadpole stage, larvae formation stage and first-stage larvae (L1) and counted in each well under a light microscope. The proportion of the L1 to other stages was determined.

Anthelmintics tested

The anthelmintics, pyrantel tartrate (Pfizer) and levamisole hydrochloride (Aldrich), were first dissolved in the culture medium to prepare the stock solution at the concentration of 10^{-3} g ml^{-1} (5×10^{-3} M). The concentrations reported refer to the weights of the salts.

Effects of anthelmintics on egg development

No significant difference in egg development was observed when 100 μl or 200 μl culture medium was used, so 100 μl was used in all subsequent experiments. Twenty microlitres of culture medium containing about 20 eggs were transferred to each well containing 80 μl of culture medium, to which was added the stock solution of the drug to yield concentrations from 10^{-10} to 10^{-4} g ml^{-1}. Seven to ten wells on each concentration of the anthelmintic were used. The plates were maintained at 37°C under 5% CO_2 for 1 week; exposure to the drugs was maintained throughout the course of the experiments. Developmental stages in each well were counted and the proportion of the L1 to other stages was determined.

Effects of drug exposure time on egg development

To examine whether drug effects are reversible, eggs were treated with drugs at 10^{-8} or 10^{-4} g ml^{-1} for 1 or 3 h, and then washed three times with culture medium and maintained in 100 μl culture medium without the drug. The plates were kept at 37°C under 5% CO_2 for 1 week. Developmental stages in each well were counted and the proportion of the L1 to other stages was determined.

Statistical analysis

The mean values were compared by the Mann-Whitney *U*-test, with *P* values less than 0.05 considered significant.

Results

Influence of medium volume on egg development

Eggs of *A. costaricensis* were cultured in two volumes of the culture medium, 100 μl and 200 μl, for 1 week. There was no significant difference in egg development between the two volumes tested. Eggs in both volumes developed to the larva formation stage, then hatched to L1 during 1 week of observation; 82.7% in 100 μl and 75.1% in 200 μl.

Effects of drugs on egg development

Concentration-dependent inhibition of development was caused by pyrantel tartrate (10^{-9}–10^{-8} g ml^{-1}) (5×10^{-9}–5×10^{-8} M) and levamisole hydrochloride (10^{-9}–10^{-8} g ml^{-1}) ($P < 0.05$), and none of the eggs developed to the first-stage larvae when exposed to higher concentrations of these anthelmintics (10^{-7} g ml^{-1} or more) (figs 1 and 2). Inhibition of development due to the anthelmintics was concentration- and time-dependent (figs 3 and 4).

Discussion

The increasing incidence of anthelmintic resistance in nematodes that infect livestock has initiated the development of several useful *in vitro* tests to screen for anthelmintic resistance. The most commonly used assays are for detecting resistance in gastro-intestinal nematodes (Maingi *et al*., 1998). From the comparison of *in vitro* tests for determining anthelmintic resistance, the larval development test was observed to be the most sensitive to measure quantitatively a degree of resistance in nematodes (Varady & Corba, 1999). *Angiostrongylus costaricensis* causes abdominal granulomata, the main characteristics of which include thickening and hardening of the oedematous intestinal wall with a miliary, yellowish inflammation induced by eggs at various stages of development. Thus the effects of anthelmintics on egg development are considered useful to obtain information on new strategies for chemotherapy. An *in vitro* cultivation system for developing eggs to L1 of *A. costaricensis*, a tissue-dwelling nematode, was established by Hata (1996), who observed that 34% of eggs developed to L1 in Ham's F-12 medium under 8% CO_2 by 10 days of cultivation. The addition of serum to the medium did not affect the rate of development of the eggs. In the present study, about 80% of eggs developed to L1 in the same medium, supplemented by 10% foetal calf serum under 5% CO_2 by 7 days. Further investigations on the effects of CO_2 and serum on egg development are necessary to optimize this cultivation system for examining the pathology, biochemistry and immunology of *A. costaricensis*.

Since *A. costaricensis* develops to the adult stage and eggs deposited in mesenteric arteries are known to be an aetiologic agent in humans (Loria-Cortes & Lobo-Sanahuja, 1980), studies of the effects of anthelmintics on both eggs and adult worms are warranted. *In vitro* experiments revealed that these anthelmintics caused a complete spastic paralysis of adult female worms of *A. costaricensis* at concentrations of 10^{-7} M (2×10^{-8} g ml^{-1}) (Terada *et al*., 1986). Results of the present study show that pyrantel and levamisole at 10^{-7} g ml^{-1} inhibit the development of cultured eggs. Drug concentrations showing detectable effects on adult motility and egg development were similar. However, eggs cultured with

https://doi.org/10.1017/S0022149X00701532 Published online by Cambridge University Press

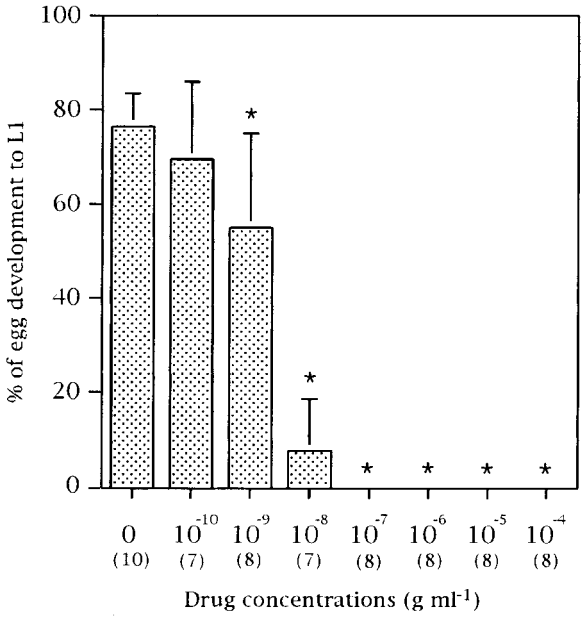

Fig. 1. Effects of pyrantel on egg development of *Angiostrongylus costaricensis*. Columns and bars represent means + SD. Numbers in parentheses represent the number of wells examined for each anthelmintic concentration. *Significantly lower than the value of control experiment (0 g ml^{-1}) (Mann-Whitney *U*-test, $P < 0.05$).

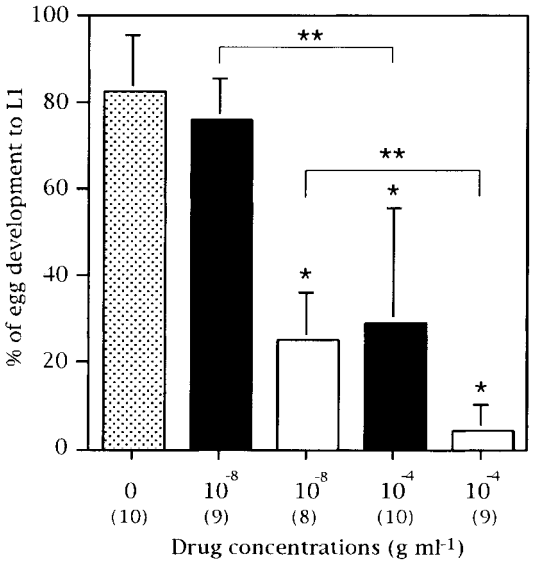

Fig. 3. Effects of exposure time of pyrantel on egg development of *Angiostrongylus costaricensis*. Columns and bars represent means + SD. Numbers in parentheses represent the number of wells examined for each anthelmintic concentration. *Significantly lower than the value of control experiment (0 g ml^{-1}) (Mann-Whitney *U*-test, $P < 0.05$). **Significant difference between 1 h (■) and 3 h (□) groups at the same concentration of anthelmintic (Mann-Whitney *U*-test, $P < 0.05$).

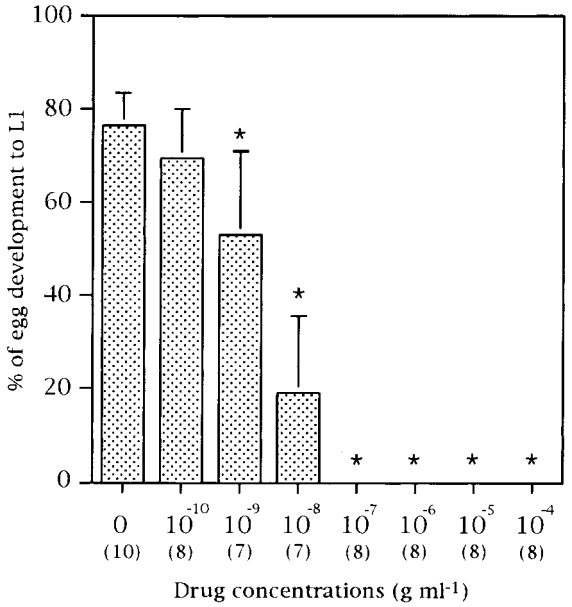

Fig. 2. Effects of levamisole on egg development of *Angiostrongylus costaricensis*. Columns and bars represent means + SD. Numbers in parentheses represent the number of wells examined for each anthelmintic concentration. *Significantly lower than the value of control experiment (0 g ml^{-1}) (Mann-Whitney *U*-test, $P < 0.05$).

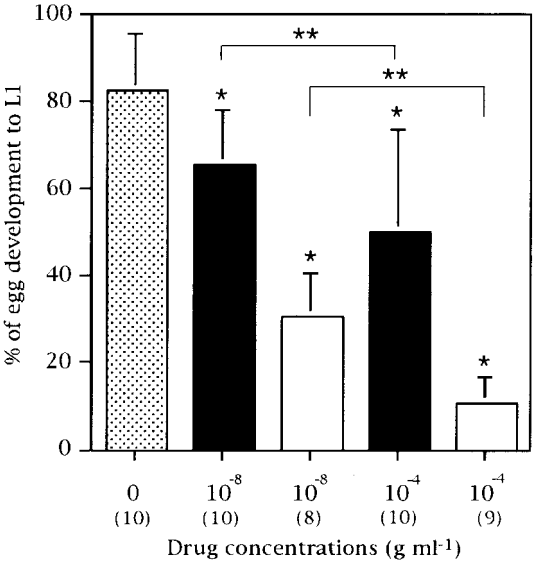

Fig. 4. Effects of exposure time of levamisole on egg development on *Angiostrongylus costaricensis*. Columns and bars represent means + SD. Numbers in parentheses represent the number of wells examined for each anthelmintic concentration. *Significantly lower than the value of control experiment (0 g ml^{-1}) (Mann-Whitney *U*-test, $P < 0.05$). **Significant difference between 1 h (■) and 3 h (□) groups at the same concentration of anthelmintic (Mann-Whitney *U*-test, $P < 0.05$).

https://doi.org/10.1017/S0022149X00701532 Published online by Cambridge University Press

pyrantel or levamisole at 10^{-4} g ml^{-1} (5×10^{-4} M) for 1 week developed to the tadpole stage or cleavage stage, respectively (data not shown).

Pyrantel is widely used as a single oral dose in the treatment of ascariasis and enterobiasis. A pharmacological property of pyrantel is that it induces depolarizing neuromuscular-blockade (Aubry *et al.*, 1970). Levamisole is used for a broad range of nematode infections, and is widely used to treat ascariasis. Levamisole induces spastic contraction of larval and adult nematodes (Van den Bossche, 1976). Pharmacological experiments have demonstrated that levamisole acts as a ganglion-stimulating compound that induces a neuromuscular inhibition of the depolarizing type (Coles, 1977). Levamisole also interacts with the fumarate reductase system in the mitochondria of nematodes. This system plays a key role in various parasitic worms (Van den Bossche, 1980). Further studies are necessary to clarify the mechanisms of inhibitory effects of these anthelmintics on egg development in *A. costaricensis*.

In the present study, cultivation with pyrantel or levamisole for 1 or 3 h inhibited egg development, indicating that this effect is irreversible. Pyrantel given orally is slightly absorbed from the intestine (Aubry *et al.*, 1970). When given orally in very large doses, toxic effects are produced, and hence this drug is commonly used to treat nematodes living the gastrointestinal tract. In contrast, levamisole is rapidly absorbed from the gastrointestinal tract, but its therapeutic doses are devoid of measurable side effects in mammals. In man peak plasma levels of about 7×10^{-7} g ml^{-1} (3.5×10^{-8} M) are reached within 1–2 h after oral administration of a single dose of 150 mg (Graziani & de Martin, 1977). This drug has a plasma half-life of about 4 h and is eliminated from the body within 2 days. Thus, the inhibitory effect of levamisole on egg development should suppress granuloma formation and transmission of this disease, by preventing the spread of infection to intermediate hosts.

References

Aubry, M.L., Cowell, P., Davey, M.J. & Shevde, S. (1970) Aspects of the pharmacology of a new anthelmintic: pyrantel. *British Journal of Pharmacology* **38**, 332–344.

Coles, G.C. (1977) The biochemical mode of action of some modern anthelmintics. *Pesticide Science* **8**, 536–543.

Freshney, R.I. (1987) Maintenance of the culture-cell lines. pp. 127–136 in *Culture of animal cells. A manual of basic technique*. New York, Alan R. Liss, Inc.

Graziani, G. & Martin, G.L. de (1977) Pharmacokinetic studies on levamisole. *Drugs under Experimental and Clinical Research* **2**, 221–223.

Hata, H. (1996) *In vitro* cultivation of *Angiostrongylus costaricensis* eggs to first stage larvae in chemically defined medium. *International Journal for Parasitology* **26**, 281–286.

Ishih, A. (1994) Die Wirkung des Anthelminthikums Levamisol auf erste *Angiostrongylus cantonensis*-Larven in infizierten Ratten. *Parasitology Research* **80**, 267–270.

Maingi, N., Bjorn, H. & Dangolla, A. (1998) The relationship between faecal egg count reduction and the lethal dose 50% in the egg hatch assay and larval development assay. *Veterinary Parasitology* **77**, 133–145.

Morera, P. (1973) Life history and redescription of *Angiostrongylus costaricensis* Morera and Cespedes, 1971. *American Journal of Tropical Medicine and Hygiene* **22**, 613–621.

Morera, P. (1985) Abdominal angiostrongyliasis: a problem of public health. *Parasitology Today* **1**, 173–175.

Loria-Cortes, R. & Lobo-Sanahuja, J.F. (1980) Clinical abdominal angiostrongylosis. A study of 116 children with intestinal eosinophilic granuloma caused by *Angiostrongylus costaricensis*. *American Journal of Tropical Medicine and Hygiene* **29**, 538–544.

Terada, M., Rodriguez, B.O., Dharejo, A.M., Ishii, A.I. & Sano, M. (1986) Studies on chemotherapy of parasitic helminths (XXVI). Comparative *in vitro* effects of various anthelmintics on the motility of *Angiostrongylus costaricensis* and *A. cantonensis*. *Japanese Journal of Parasitology* **35**, 93–95.

Terada, M., Tungtrongchitr, A., Ishih, A. & Sano, M. (1992) Effects of membendazole on abdominal angiostrongyliasis in mice after worm maturation: preliminary trials. *Japanese Journal of Parasitology* **41**, 481–486.

Tungtrongchitr, A., Ishih, A., Terada, M. & Radomyos, P. (1992) The influence of immunosuppression on the efficacy of mebendazole in mice infected with adult *Angiostrongylus costaricensis*. *Journal of Tropical Medicine and Parasitology* **15**, 61–70.

Van den Bossche, H. (1976) The molecular basis of anthelmintic action. pp. 553–572 *in* Van den Bossche, H. (*Ed.*) *Biochemistry of parasites and host–parasite relationships*, Amsterdam, Elsevier.

Van den Bossche, H. (1980) Peculiar targets in anthelmintic chemotherapy. *Biochemical Pharmacology* **29**, 1981–1990.

Varady, M. & Corba, J. (1999) Comparison of six *in vitro* tests in determining benzimidazole and levamisole resistance in *Haemonchus contortus* and *Ostertagia circumcincta* of sheep. *Veterinary Parasitology* **80**, 239–249.

(Accepted 29 December 2000)