Effect of praziquantel on the strobilar development of *Mesocestoides corti* in vitro

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Abstract

The effect of praziquantel (PZQ) on the strobilar development of the cyclophyllidean cestode *Mesocestoides corti* was explored. *Mesocestoides corti* larvae were cultivated under conditions reported to favour their differentiation to the adult stage. Parasites were exposed to 0.1 $\mu g\,ml^{-1}$ PZQ for 16 h and subsequently transferred to drug-free medium. The ocurrence of segmentation – an early event of the larval somatic differentiation to the adult worm – was considered as quantitative data. This phenomenon was evidenced earlier in worms transiently exposed to PZQ with respect to control cultures. Moreover, the rate of segmentation of drug-treated worms at the end of the experiment almost doubled that of control worms. To date, no similar effect on any cestode developmental process has been reported for an anthelmintic drug. In the light of the existing knowledge and understanding of PZQ mechanisms of action, the proposed experimental approach could contribute to the elucidation of pathways and mechanisms involved in cestode strobilar development.

Introduction

Difficulties encountered in attaining effective control of parasitic diseases stress the need for a deeper knowledge on parasite biology, particularly of the mechanisms involved in their differentiation and adaptation processes.

Over the last decades, parasite chemotherapy has attracted considerable attention and extensive information is available about the chemistry and mode of action of many anthelmintic drugs. Moreover, their effects on distinct developmental stages of several helminth species in *in vivo* and *in vitro* systems have been described (Thomas & Gonnert, 1978; Andrews & Thomas, 1979; Thompson *et al.*, 1986; Morris *et al.*, 1987; Chappell, 1988). Little has been analysed, however, about their incidence on differentiation processes leading from one developmental stage to another.

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The introduction of *in vitro* culture techniques has permitted the analysis of different effectors on parasite development under controlled conditions. In that sense, numerous workers have determined the experimental conditions which favour the strobilar development of several cestode species (Smyth, 1990), in particular *Echinococcus granulosus* and *Mesocestoides corti* (Barrett *et al.*, 1982; Thompson *et al.*, 1982; Smyth, 1987).

Mesocestoides corti exhibits much potential as a model for the study of the biochemistry, physiology and differentiation of cestodes. Its larval form (tetrathyridium) is readily maintained in the laboratory by intraperitoneal passage through mice, providing a suitable *in vivo* study system, as well as yielding significant quantities of homogeneous parasitic material for *in vitro* assays.

We report here the effects of a sublethal concentration of praziquantel (PZQ), an extensively studied anthelmintic drug, on an early event of *M. corti* strobilar development in an *in vitro* system. In the light of the existing knowledge on mechanisms of action of PZQ, the proposed experimental design could contribute to the study of mechanisms involved in parasite developmental processes.

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Materials and methods

Care and handling of experimental animals

Experimental protocols used with the animals (mice, rats) have been approved by the Animal Care Committee of the Faculty of Sciences, according to the guidelines of the Canadian Council of Animal Care.

Maintenance of parasites

Tetrathyridia of *M. corti* (kindly provided by Dr Jack Chernin, Portsmouth, UK) were mantained by intraperitoneal passage in CD1 mice and Wistar rats (one passage through rat every four passages through mice). Two-month-old males were inoculated with *c*. 200 larvae in saline in both cases.

Collection of tetrathyridia

Two to six months post-infection, mice were killed by cervical dislocation and larvae were recovered aseptically from the peritoneal cavity in a laminar flow chamber with a Pasteur pipette and transferred to a 50 ml culture flask with pre-warmed Hank's balanced salt solution (HBSS) containing 50 $\mu g\,ml^{-1}$ gentamycin. They were rinsed once in *c*. 40 ml of fresh HBSS for about 15 min at 38°C. No further pre-treatment was implemented prior to the onset of cultivation.

In vitro *culture*

The monophasic culture protocol described by Thompson *et al.* (1982) was adopted, introducing the following modifications: no enzymatic pretreatment of the larvae was implemented and cultures were maintained under a gas phase of 5% CO_2 in air at 38°C. CMRL-1066 culture medium (Gibco) was used, supplemented with 4.2 $g\,l^{-1}$ sodium bicarbonate (Fluka), 5.2 $g\,l^{-1}$ HEPES (Sigma), 4.5 $g\,l^{-1}$ yeast extract (Oxoid), 4.3 $g\,l^{-1}$ D-glucose (Sigma) and 50 $\mu g\,ml^{-1}$ gentamycin (Herix). Medium pH was adjusted at 7.6. Half of the spent medium was replaced every 2–3 days. The number of worms was settled at 30 ± 10 per well, in 2 ml of culture medium in 24× multiwell plates (Nunc). Four different heat inactivated fetal bovine sera (two lots from Gibco, one from Sigma and one from Santa Elena Laboratory (Uruguay)) were used in parallel experiments, at a final concentration of 20%. Cultures were monitored for 12 days, using an inverted optical microscope (Olympus, model IX70).

Exposure to praziquantel (PZQ)

The drug was solubilized in dimethyl sulphoxide (DMSO) and added at the onset of cultivation, in the presence or absence of fetal bovine serum. The final concentration of PZQ was 0.1 $\mu g\,ml^{-1}$ and the final concentration of solvent never exceeded 0.5%. After 16 h of exposure to the drug, each culture well was rinsed three times with 1 ml of CMRL-1066, and the larvae transferred to a drug-free medium containing fetal serum and cultured as described above. Control worms were exposed to DMSO 0.5% for 16 h and subjected to the same treatment.

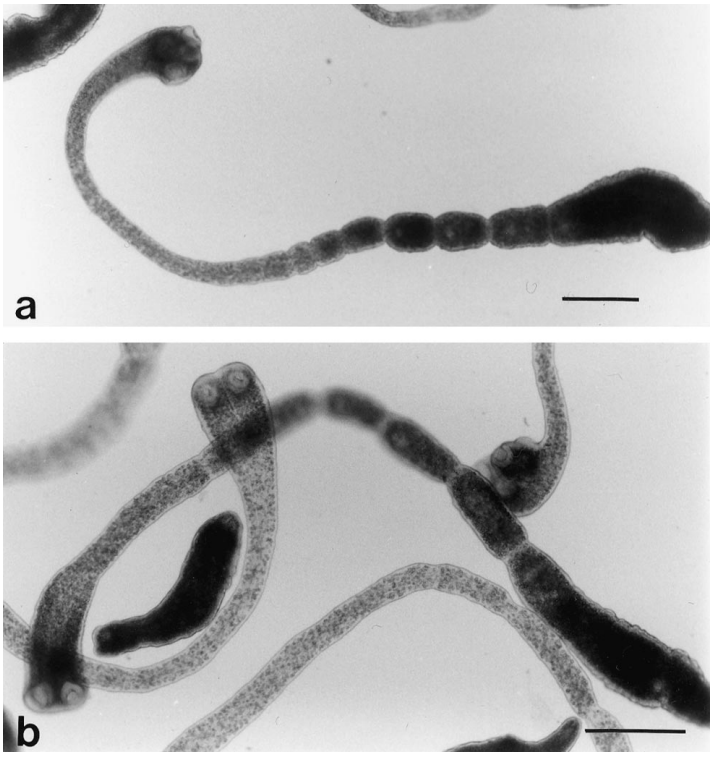

Fig. 1. *Mesocestoides corti* segmented worms at day 12 post-initial culture: (a) control specimen (b) PZQ-treated worm. Scale bar = 400 μm.

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Statistical determination of segmentation rates

The number of segmented worms from individual culture wells was registered separately on days 7, 8, 9, 11 and 12 post initial culture. Although segmentation was also observed in worms that were undergoing longitudinal fission, only specimens with four suckers were taken into account. Considering the total number of parasites per well (always within the 30 ± 10 range), percentages of segmented worms were calculated. Included into the segmented category were those specimens showing external infolding of the tegument which delineates different proglottids, as shown by optical examination. Percentages of segmentation from control and praziquantel-exposed individual cultures (24 replicates per condition) were compared by means of the Student's t-test (a probability greater than 0.01 was not considered significant).

Results

Mesocestoides corti tetrathyridia were cultivated under conditions reported to allow the strobilar development of the parasite, as described above. Under such conditions, segmented parasites were observed as early as day 6 post-initial culture (fig. 1a). Several proglottids appeared simultaneously, reaching a maximum of 15 to 20 per worm. Some specimens were analysed at day 12, by means of whole-mounted staining with Schneider's carmine and optical microscopy (OM). An early stage of sexual maturation was observed, characterized by the presence of testes and the rudiment of the cirrus pouch (not shown).

In order to analyse the effect of PZQ on strobilar development, parasites were transiently incubated in the presence of the drug at the onset of cultivation. As seen at the OM level, a 16 h exposure to 0.1 μg ml^{-1} PZQ resulted in distended specimens of irregular shape,

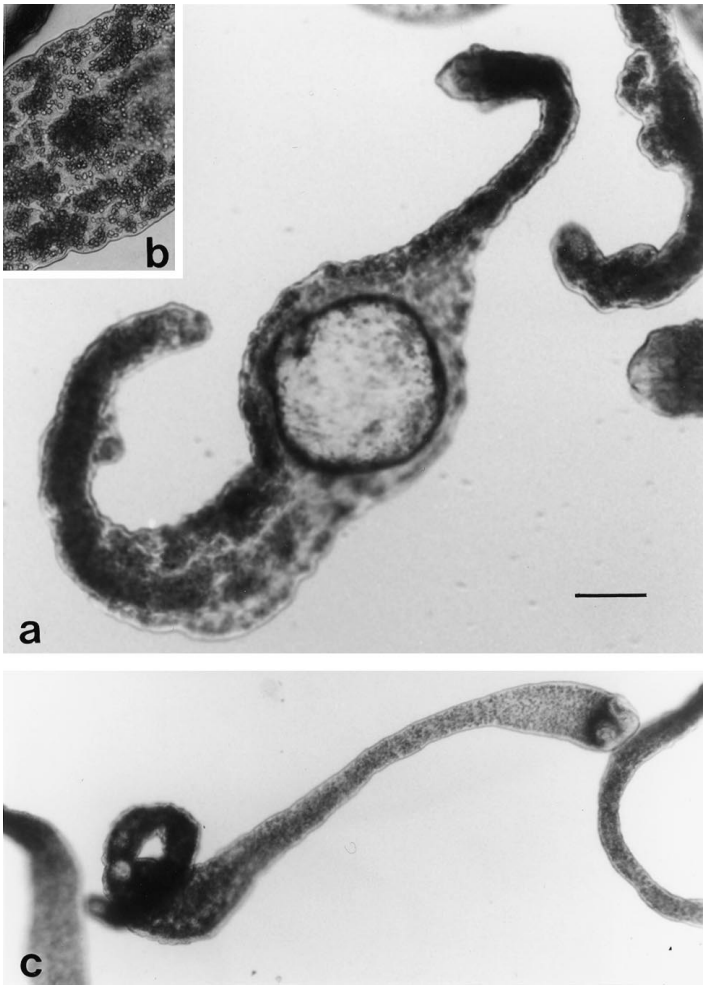

Fig. 2. *In vitro* effect of praziquantel (PZQ) on *Mesocestoides corti* tetrathyridia. (a) Tetrathyridium exposed to 0.1 μg ml^{-1} PZQ for 16 h, showing the formation of extensive vesicles (scale bar = 200 μm). A detailed image of tegument striated appearance is shown in (b) (scale bar = 350 μm). (c) Tetrathyridium at day 6 post initial culture. This specimen was exposed to PZQ as described in (a) and subsequently transferred to a drug-free medium.

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striated appearance and reduced motility, with the exception of the suckers. Some specimens also developed body vesicles (fig. 2a and b). To preclude a possible osmotic effect on the tegument unrelated to the drug action, an experiment was included in which sodium chloride was added to give the same final osmolarity obtained with the drug. No significant morphological alteration was observed under such conditions (not shown).

Following exposure to the drug, parasites were transferred to a drug-free medium and cultivated under the conditions described. After 1–2 days in a drug-free medium, worms that had been exposed to PZQ often presented posterior ends darker and more irregular in shape than control ones. Nonetheless, they generally regained their normal tegumental morphology and motility even when vesicles had developed (fig. 2c). Cultures were monitored for as long as 18 days, but both control and treated worms showed signs of deterioration after day 12.

The described dependent variable regarded as quantitative data is the rate of segmentation of drug-treated and control specimens, studied over a 12-day time span. On worms transferred to a drug-free medium, segmentation was observed at least a day earlier than in control specimens. Morphology of strobilating worms from drug-exposed and control groups did not differ significantly (fig. 1a and 1b). Moreover, the maximum final percentage of segmentation achieved under the experimental conditions was also higher for drug-treated worms. The rate of segmentation of drug-treated worms on day 12 almost doubles that of control worms (fig. 3). These results were corroborated in three additional independent experiments (data not shown).

The dependence of PZQ-stimulated segmentation on different fetal bovine sera was also considered. Comparable results were systematically obtained with three of the four different sera utilized (fig. 3).

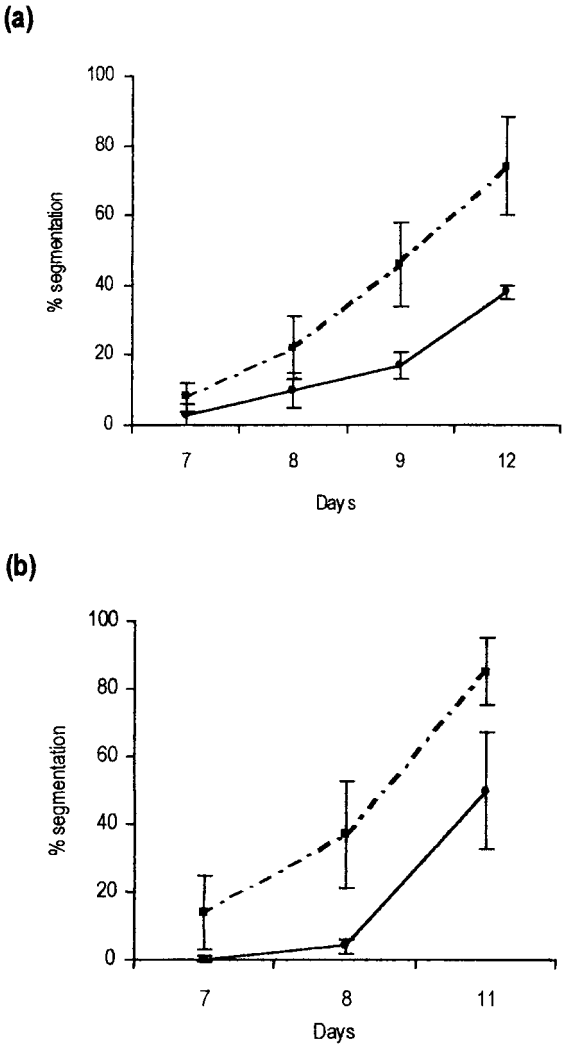

Fig. 3. Effect of praziquantel (PZQ) on *Mesocestoides corti* segmentation rate. Tetrathyridia were exposed to 0.1 μg ml^{-1} PZQ solubilized in 0.5% DMSO for 16 h, then transferred to drug-free medium and cultured for a 12-day period. Percentages of segmented worms were registered for individual wells and data subjected to statistical analysis (not shown). (a) and (b) represent two independent experiments using different fetal bovine sera (Gibco lot No. 39N6266 and Santa Elena-URUGUAY, respectively). –·–■–·– PZQ; —●— control.

Discussion

The nature of the factors and signal pathways involved in cestode developmental processes are not accounted for to date, thus constituting a fertile field of fundamental research, especially in the design of alternative control strategies.

In vitro cultivation is a most powerful tool which permits an analysis of putative effectors (nutritional factors, host hormones, etc.) on parasite development under controlled conditions. We have reproduced an *in vitro* culture system initially devised to obtain sexual maturation of *E. granulosus* (Smyth, 1967) and reported to favour the strobilar development of *M. corti* (Barret *et al.*, 1982; Thompson *et al.*, 1982; Ong & Smyth, 1986). Parasite strobilization proceeded as described by Thompson *et al.* (1982) until day 12, after which degeneration of parasites was observed and cultures were discontinued. Evidently, the changing nutritional requirements of developing worms were not being catered for by the utilized culture medium. Although we were unable to obtain fully mature egg producing worms, the early stages of strobilar development leading to segmentation and the formation of rudimental genitalia were successfully reproduced. Segmentation times and morphology of segmented worms within the experiment time span were similar to those described by Thompson *et al.* (1982).

Segmentation as defined in Mehlhorn *et al.* (1981) and Thompson (1995) comprises the external infolding of tegument which demarcates each developing proglottid and gives rise to the characteristic constricted appearance of strobilated worms. Although the formation of rudimentary genital structures was observed in treated and untreated segmented worms, the effect of PZQ on proglottization was not statistically analysed.

Anthelmintic drugs must be placed among the effectors that deserve attention concerning their possible incidence on parasite development. Praziquantel, in

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particular, is the drug of choice for the treatment of a wide range of veterinary and human helminthic infections. It has proved to be highly effective against adult worms but less so against larval forms (Heath & Lawrence, 1978; Andrews & Thomas, 1979; Thompson *et al*., 1986; Chappell, 1988).

Even if the effects of PZQ have been extensively studied both *in vivo* and *in vitro* on a wide range of helminths, its effects on parasite developmental processes have not been reported so far. Our results show clearly that a transient exposure of *M. corti* larvae to 0.1 $\mu g\,ml^{-1}$ PZQ stimulates subsequent worm segmentation, an early event of somatic differentiation.

As with many other described effects of PZQ on cestodes and trematodes (Martin, 1997), the stimulation of *M. corti* segmentation by transient exposure to the drug could be explained by an increased calcium membrane permeability. In particular, PZQ has been shown to stimulate the release of endogenous Ca^{2+} from *Hymenolepis diminuta* (Pritchard *et al*., 1982). On the other hand, PZQ reportedly causes a rapid and dramatic increase in Ca^{2+} influx across the tegumental membrane of the trematodes *Schistosoma mansoni* (Pax *et al*., 1978; Fetterer *et al*., 1980), *S. japonicum* (Pax *et al*., 1978) and *Opisthorchis viverrini* (Ruenwongsa *et al*., 1983). Although it remains a controversial matter, it has been proposed that Ca^{2+} mobilization in *S. mansoni* and *M. lineatus* could lead to the activation of signal transduction pathways involving phospholipase C and protein kinase C (Kawamoto *et al*., 1986; Martin, 1997). As there is no report on such metabolic pathways in tapeworms, further studies into PZQ influence on Ca^{2+} mobilization and strobilar development of *M. corti* would provide useful evidence on the ontogenic processes of cestodes.

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