Localization, Specific Activity, and Molecular Forms of Acetylcholinesterase in Developmental Stages of the Cestode *Mesocestoides corti*

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The nervous system of flatworms is quite simple although there is increasing evidence indicating that it is chemically complex. Studies of the nervous system in these animals have only been performed in the larval stage or in the adult worms, which are easy to obtain in nature, while the description of the nervous system in developing stages of these organisms is missing. *Mesocestoides corti* is a parasitic platyhelminth whose larvae can be induced in vitro to develop to adult, sexually mature worms, opening the possibility of studying the nervous system of a flatworm in different stages of development. Here, we describe the presence, activity, location, and molecular forms of acetylcholinesterase (AChE) in different stages of development of *M. corti*, from the larvae to adult forms of this endoparasite, obtained in in vitro cultures after induction of the larval stage with trypsin. Our results point to AChE as a molecular marker of the nervous system in platyhelminthes. The change in molecular forms of this enzyme and the increase in its activity during development from larvae to adult worm may reflect the presence of a more complex nervous system, necessary to adjust and coordinate the movement of a much bigger structure. A relationship between the development of the reproductive apparatus in segmented and adult worms with a more complex nervous system in these stages is also apparent. Finally, our study opens the possibility of applying anti-AChE as more effective therapeutic strategies against cestode parasites.

Platyhelminthes are metazoans phylogenetically unique because they corresponded apparently to be the basal stock from which the majority of the higher forms of life, including mammals, have evolved (Hyman, 1951; Adoutte et al., 2000). Most probably, they are the first animals to display bilateral symmetry, cephalization, and condensation of disperse neuronal elements into a whole central nervous system (CNS), as well as well developed muscles (Pax et al., 1996). Considering the low number of neurons, the nervous system of flatworms is quite simple, however, there is increasing evidence indicating that it is neurochemically complex (Gustafsson, 1992; Halton and Gustafsson, 1996; Hrckova et al., 1994, 2004) and that their cerebral ganglia present a high level of molecular regionalization (Cebrià et al., 2002). Moreover, a comparison of nucleotide sequences from EST clones obtained from the head portion of the planarian *Dugesia japonica* with ORFs of human, *Drosophila* and *C. elegans* provided evidence at the molecular level for the existence of a common ancestral CNS (Mineta et al., 2003), emphasizing the importance of studying this system in platyhelminthes. Studies of the nervous system in these animals have only been performed in the larval stage or in adult worms of different species, which are easy to obtain in nature, or to maintain in vitro (Geary et al., 1992), while the description of the nervous system in developing stages of these organisms is missing. Morphological, biochemical, and molecular biology approaches to the study of endoparasites during their development present extreme difficulties due to the complex and indirect life cycles of these organisms, which are usually impossible to reproduce in vitro (Geary et al., 1992).

Mesocestoides corti is a member of the Platyhelminth phylum and corresponds to the class Cestoda, order Cyclophylidea, family Mesocestoididae. It presents a complex biological cycle, the first intermediary host being an oribatid insect, the secondary host a rodent or a reptile or a bird, and the definitive host a carnivorous or omnivorous mammal, including man (Specht and Voge, 1965; Hess and Guggenheim, 1977; Barret et al., 1982; Barriga, 1997; von Nickisch-Rosenegk et al., 1999). In the secondary hosts, the larval form or tetrathyridium is formed, which reproduces asexually in the peritoneal cavity infecting the liver and other organs (White et al., 1982; Barriga, 1997). In the definitive hosts, ingested tetrathyridia attach the intestinal mucosa and develop to the adult mature-egg producing worms. The eggs are

Contract grant sponsor: Fondecyt; Contract grant numbers: 1010817, 1050135; Contract grant sponsor: Network for Research and Training in Parasitic Diseases, SIDA/SAREC; Contract grant number: 2003/2005.

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eliminated with the faeces and ingested by the oribatid insects, thus closing the biological cycle of this parasite.

The use of *M. corti* as a research model for experimental studies in cestode biology has been recognized since it was first reported that the second larval (metacestode) stage, or tetrathyridia, was capable of multiplying asexually in the peritoneal body cavity of mice, where they could be maintained indefinitely by serial passage (Specht and Voge, 1965; Hart, 1968). Furthermore, it was found that treatment of *M. corti* tetrathyridia with trypsin in in vitro cultures induced the development of these larval forms to adult, sexually mature worms (Kawamoto et al., 1986a,b; Espinoza, 2002; Markoski et al., 2003; Espinoza et al., 2004), opening the possibility of studying the nervous system of a cestode during development, from the larval stage to the mature adult form.

The neurotransmitters acetylcholine (ACh) (Hart, 1967), 5-hydroxytryptamine (Hrckova et al., 1994), neuropeptide F (Hrckova et al., 1993), and nitric oxide (NO) (Terenina et al., 1999) have been localized in tetrathyridia forms of *M. corti*. However, studies aiming at the characterization of the nervous system in different stages of the developing larvae leading to the adult worms are missing. ACh is considered the principal inhibitory neurotransmitter in the neuromuscular and nervous system in platyhelminthes (Brownlee and Fairweather, 1996; Halton and Gustafsson, 1996; Giménez-Pardo et al., 2000). Acetylcholinesterase (AChE; EC 3.1.1.7) is the enzyme involved in the hydrolysis of ACh in peripheral and central synapses (Fernández et al., 1996; Soreq and Seidman, 2001). In vertebrates, the activity and molecular forms of AChE are regulated during development (Moreno et al., 1998), presenting an increase in specific activity and the appearance of more complex molecular forms of this enzyme (Taylor and Radic, 1994; Fernández et al., 1996). As a contribution to the neurobiology of cestodes, we describe the presence, localization, specific activity, and molecular forms of AChE in different stages of development of the endoparasite *Mesocestoides corti*, from tetrathyridia to adult worms, obtained in in vitro cultures after induction of the larval stage with trypsin.

MATERIALS AND METHODS

Maintenance of *Mesocestoides corti* tetrathyridia in mice

Female, 3 months old Balb/c mice, bred in the laboratory, were used between 3 and 4 months old weighing 25–30 g. The animals were kept on a natural light and dark schedule and fed ad libitum. Mice were inoculated intraperitoneally with 50 μl tetrathyridia sedimented by gravity, resuspended in 100 μl phosphate-buffered saline (PBS), pH 7.2, 75 U/ml penicillin and 75 μg/ml streptomycin. After 3 months in the mice room, they were killed by cervical dislocation. The tetrathyridia were collected from the peritoneal cavity using standard aseptic techniques and washed in PBS at 37°C.

Induction of development of *Mesocestoides corti* tetrathyridia to adult worms

M. corti tetrathyridia were incubated in RPMI 1640 (GIBCO BRL, Life Technologies, Grand Island, NY) culture medium, containing 20% (v/v) heat inactivated fetal bovine serum (FBS), 75 U/ml penicillin, 75 μg/ml streptomycin and 1×10^5 BAEE (N-benzoil-L-arginine ethyl ester) units of trypsin/ml for 24 h at 37°C under a CO_2 5% partial pressure. After this time, the medium was replaced with the same medium but without trypsin and changed every 2 days to avoid acidification. Segmentation of tetrathyridia starts between days 4 and 5 post-treatment with trypsin and ends around day 10, when adult, sexually mature worms can be observed.

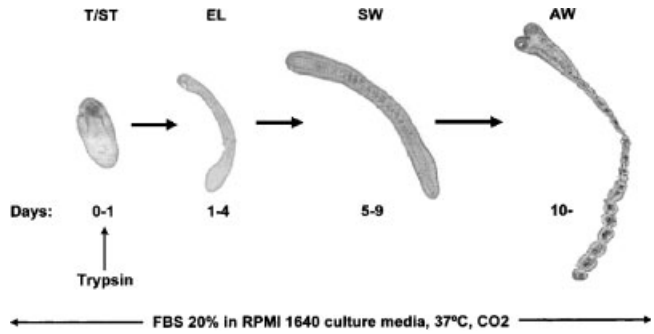

Fig. 1. Induction of development of *Mesocestoides corti* tetrathyridia to adult worms. *M. corti* tetrathyridia were incubated in RPMI 1640 (GIBCO) culture medium, containing 20% (v/v) heat inactivated fetal bovine serum (FBS), 75 U/ml penicillin, 75 μg/ml streptomycin, and 1×10^5 BAEE units of trypsin/ml for 24 h at 37°C under 5% CO_2 partial pressure. After this time, the medium was replaced for the same medium but without trypsin and changed every 2 days to avoid excessive acidification. T/ST, stimulated larvae; EL, elongated larvae; SW, segmented worms; AW, adult worms.

Based on morphological observations under a light microscope and following previous descriptions (Espinoza, 2002; Espinoza et al., 2004), tetrathyridia treated for 24 h with trypsin were considered as stimulated larvae while parasites between 1 and 4 days post-treatment with trypsin, were taken as elongated larvae; these last forms are clearly longer than tetrathyridia but do not present segmentation. From day 5 to day 9 after trypsin induction of development, the process of segmentation is evident but a reproductive apparatus is missing or not developed; these parasites were considered segmented. Finally, parasites from day 10 after trypsin induction were considered adult worms, presenting developed reproductive organs (Fig. 1).

M. corti homogenization

Parasites at different developmental stages were homogenized manually in 20 mM PBS, pH 7.0, 1.0 M NaCl, 0.5% Triton X-100, using a glass-to-glass Potter homogenizer. The homogenates were sonicated three times during 1 min each with 2 min intervals and a potency of 60 W with a Cole Parmer CP 130 PB-1 sonicator. The homogenized and sonicated samples were centrifuged in a Sorvall–Combi ultracentrifuge at 100,000*g* during 1 h. All steps were carried out at 4°C.

Total cholinesterase, AChE, and butyrylcholinesterase (BuChE) activities

Total cholinesterase activity (TChE) was determined in the 100,000*g* supernatant of the total homogenate of the parasite by the spectrophotometric method of Ellman et al. (1961). Standard assays were performed in a 1.0 ml reaction mixture containing 0.2 M PBS pH 7.0, 0.3 mM 5.5′-dithiobis(2-nitrobenzoic acid), 0.1% Triton X-100, and 0.75 mM acetylthiocholine iodide at 4°C for 16 h. AChE was measured adding 10 mM tetraisopropyl pyrophosphoramide (iso-OMPA) to the reaction mixture, as a specific inhibitor of BuChE activity. On the other hand, BuChE activity was determined adding 10 μM BW 284c51 dibromide to the reaction mixture instead of iso-OMPA, to inhibit specific AChE activity. Absorbance was read at 412 nm in a Shimadzu UV-150-02 double beam spectrophotometer.

Sedimentation analysis of AChE forms

The molecular forms of AChE present in the tissue homogenate supernatants were analyzed by velocity sedimentation, as previously described (Moreno et al., 1998). Fifty microliter of each sample was loaded onto a linear sucrose gradient (5–20% w/v) and resolved in a Sorvall–Combi ultracentrifuge using an AH-650 rotor at 100,000*g* at 4°C for 16 h. The gradient was made up with 50 mM Tris-HCl, 1% Triton X-100, 2.5 mM EDTA, 1.0 M NaCl, and 10 mM iso-OMPA. Catalase (11.3 S) was routinely used as a marker to estimate the sedimentation coefficients of the AChE forms.

Protein assay

Protein concentration was measured by the Bradford assay (Bradford, 1976) using Sigma bovine serum albumin (grade V) as a protein standard.

Localization of AChE by histochemistry

The localization of AChE as a marker of the nervous system in *M. corti* was determined by a histochemical staining method (Karnovsky and Roots, 1964). Briefly, whole parasites or histological sections were incubated in a 1.0 ml staining solution containing 2.2 mg/ml acetylthiocholine iodide, 0.1 M acetate buffer pH 6.3, 0.1 M sodium citrate, 30 mM copper sulfate and 5 mM potassium ferricyanide at 37°C for 2 h. For histological sections, worms were fixed in 1 (w/v) paraformaldehyde in PBS pH 7.2 at 4°C for 24 h, and then embedded in paraffin. The samples were analyzed with a Nikon Optiphot microscope; the images were taken with a Kodak Digital Science DC 120 camera and processed with Corel Draw 11 software.

Histological methods

Tetrathyridia, as well as different developmental stages of the parasite and adult worms, were fixed in 1% (v/v) paraformaldehyde in PBS pH 7.2 at 4°C for 24 h, and then embedded in paraffin (all reagents from Merck KGaA, Darmstadt, Germany). Blocks were cut in 5 μm-thick sections which were stained with haematoxilin-eosin following standard methods.

Statistics

Results are expressed as the mean ± SE. The significance of differences was evaluated using ANOVA followed by Tuckey's multiple comparisons post-test.

RESULTS

Cholinesterase activity at different stages of *M. corti* development

M. corti presents cholinesterase activity at all stages of development, the main enzyme activity corresponding to AChE, with around 85% of the total cholinesterase activity present in the supernatant of *M. corti* homogenates at all developmental stages (Fig. 2). Segmented and adult forms of the parasite present approximately three times more AChE activity than the tetrathyridia larval form (Fig. 2). BuChE represents just a minor percentage of the total cholinesterase activity. Minor oscillations in AChE activity are observed from day 1 on after trypsin treatment while a sharp increases in AChE activity is evident at day 9, coincident with the beginning of the transition from segmented to adult, fully differentiated and mature worms (Figs. 1 and 3).

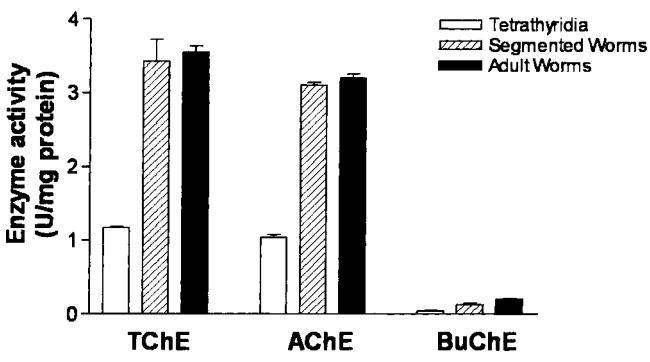

Fig. 2. Cholinesterase activity at different stages of *M. corti* development. Total cholinesterase activity (TChE) was determined by the method of Ellman et al., 1961. Enzyme activity was measured in the 100,000*g* supernatant obtained after centrifugation of the parasite total homogenate. AChE was measured adding 10 mM iso-OMPA to the reaction mixture, as a specific inhibitor of BuChE activity. On the other hand, BuChE activity was determined adding 10 μM BW 284c51 dibromide to the reaction mixture instead of iso-OMPA, to specifically inhibit the AChE activity.

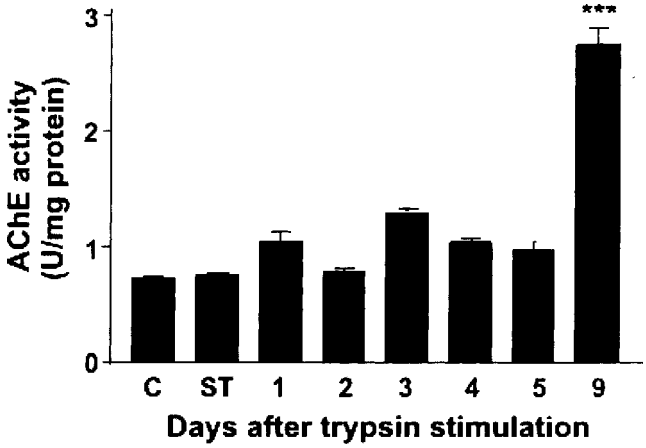

Fig. 3. AChE activity during larvae development to adult worms. AChE was determined as in Figure 2. Results correspond to three experiments $P = 0.001$. C, control not-treated tetrathyridia; ST, stimulated tetrathyridia (0–24 h after trypsin treatment).

Molecular forms of AChE in two developmental stages of *M. corti*

Velocity sedimentation analysis of the AChE present in *M. corti* tetrathyridia showed that most of the enzymatic activity was found in a peak with a sedimentation coefficient 4-6S, which corresponds to the G_1 or monomeric molecular form of the enzyme (Moreno et al., 1998). In adult worms, the same molecular form of low sedimentation coefficient (4–6 S) is present, but a more complex form (10.5 S) was also observed, which corresponds to the tetramer of the enzyme (G_4) (Fig. 4 and Moreno et al. (1998)).

Morphological localization of AChE in different developmental stages of *M. corti*

The histochemical staining of AChE in *M. corti* tetrathyridia shows an intense enzymatic activity in the anterior cellular territories of the parasite, more precisely corresponding to the pair of cerebral ganglia

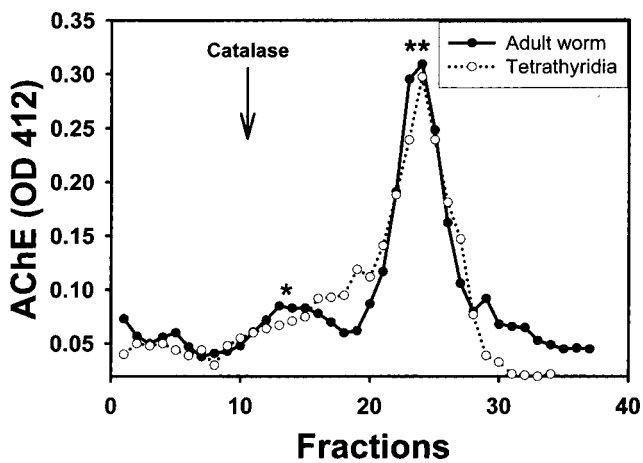

Fig. 4. Velocity sedimentation analysis of the AChE forms in *M. corti* tetrathyridia and adult worms. Fifty μl aliquots of the 100,000*g* supernatant of the parasite total homogenate were analyzed by velocity sedimentation in 5–20% sucrose gradients. The arrow indicates the sedimentation coefficient of catalase (11.3 S). G1(**): AChE monomer, sedimentation coefficient 4–6 S; G4(*): AChE tetramer, sedimentation coefficient 10.5 S.

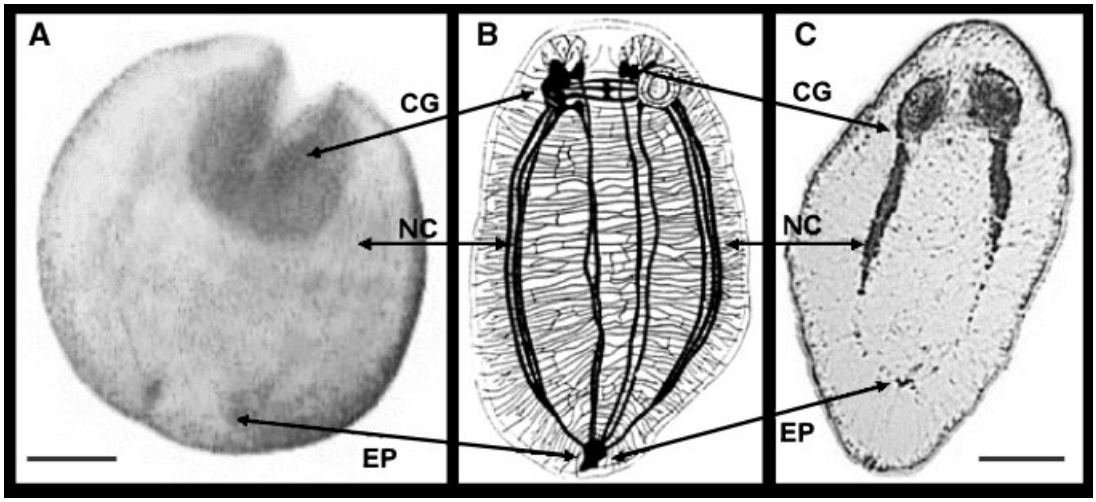

Fig. 5. AChE localization in *M. corti* tetrathyridia. AChE was detected by the method of Karnovsky and Roots (1964). Part **A**: AChE in tetrathyridium, whole mount. Part **B**: Scheme of the tetrathyridium nervous system, as Hart (1967). Part **C**: Histological section of a tetrathyridium, haematoxilin-eosin stained. CG, cerebral ganglia; EP, excretory plexus; NC, nerve cords. Bars, 100 μm.

located in the scolex (Fig. 5A,B,C). In the same figure, longitudinal nervous cords, which terminate as an excretory plexus in the caudal region, can also be seen. These observations are coincident with a previous description of the nervous system of *M. corti* tetrathyridia (Hart, 1967).

The localization of AChE in elongated larvae and segmented worms is similar to that observed in tetrathyridia, although a higher activity in the region that corresponds to the cerebral ganglia is evident in adult worms (compare Figs. 5A and 6A with 7D). Regarding the longitudinal nerve cords the location of the enzyme is more defined in segmented and adult worms than in tetrathyridia (compare Fig. 5A with Figs. 6A,B, 7B,C). The excretory plexus presents a similar cholinesterase activity in the different stages of development of the parasite (see Figs. 5A, 6A, and 7C). Of particular interest is the noteworthy activity of AChE in the genital pores of the segmented stage of development (Fig. 6A) and in the reproductive apparatus in worms at the beginning of their segmentation (Fig. 6B), which persists in the adult worm (Fig. 7B).

In Figure 7A, the presence of AChE is shown in a whole adult worm, from the scolex at the anterior region to the caudal end. In the scolex, a strong activity is associated to the suckers and cerebral ganglia (Fig. 7D). Lateral longitudinal nervous cords, which start in the ganglia (Fig. 7D), pass through the proglotids (Fig. 7B) and end in the caudal region of the parasite (Fig. 7C). In Figure 7E, a section of the scolex of the adult worm is shown; the cerebral ganglia, nerve cords, and suckers are evident.

DISCUSSION

The results of the present study provide morphological as well as biochemical evidence for the presence, activity, localization, and molecular forms of cholinesterases in the nervous system of *Mesocestoides corti*. To our knowledge, this is the first study pointing to AChE as a marker of the nervous system during development of the different forms of a cestode, that is, from the larval stage to the adult worm. Our observation that AChE is

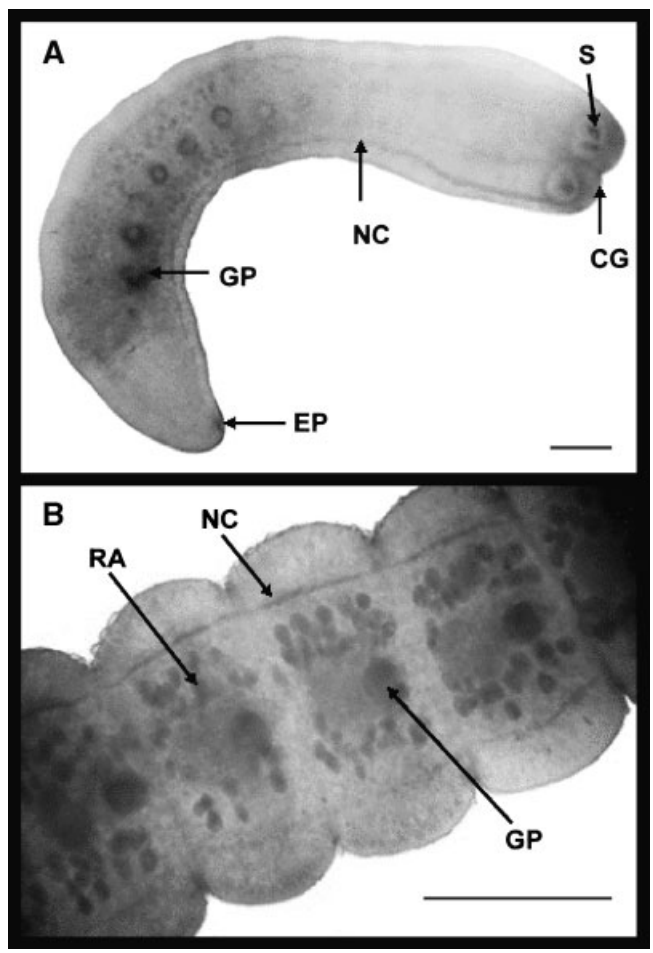

Fig. 6. AChE localization in segmented worms. AChE was detected as described in the previous figure in whole mount preparations of segmented worms. Part **A**: Complete segmented worm. Part **B**: Segments. CG, cerebral ganglia; EP, excretory plexus; GP, genital pore; RA, reproductive apparatus; S, suckers; SC, scolex; NC, nerve cord. Bars, 100 μm.

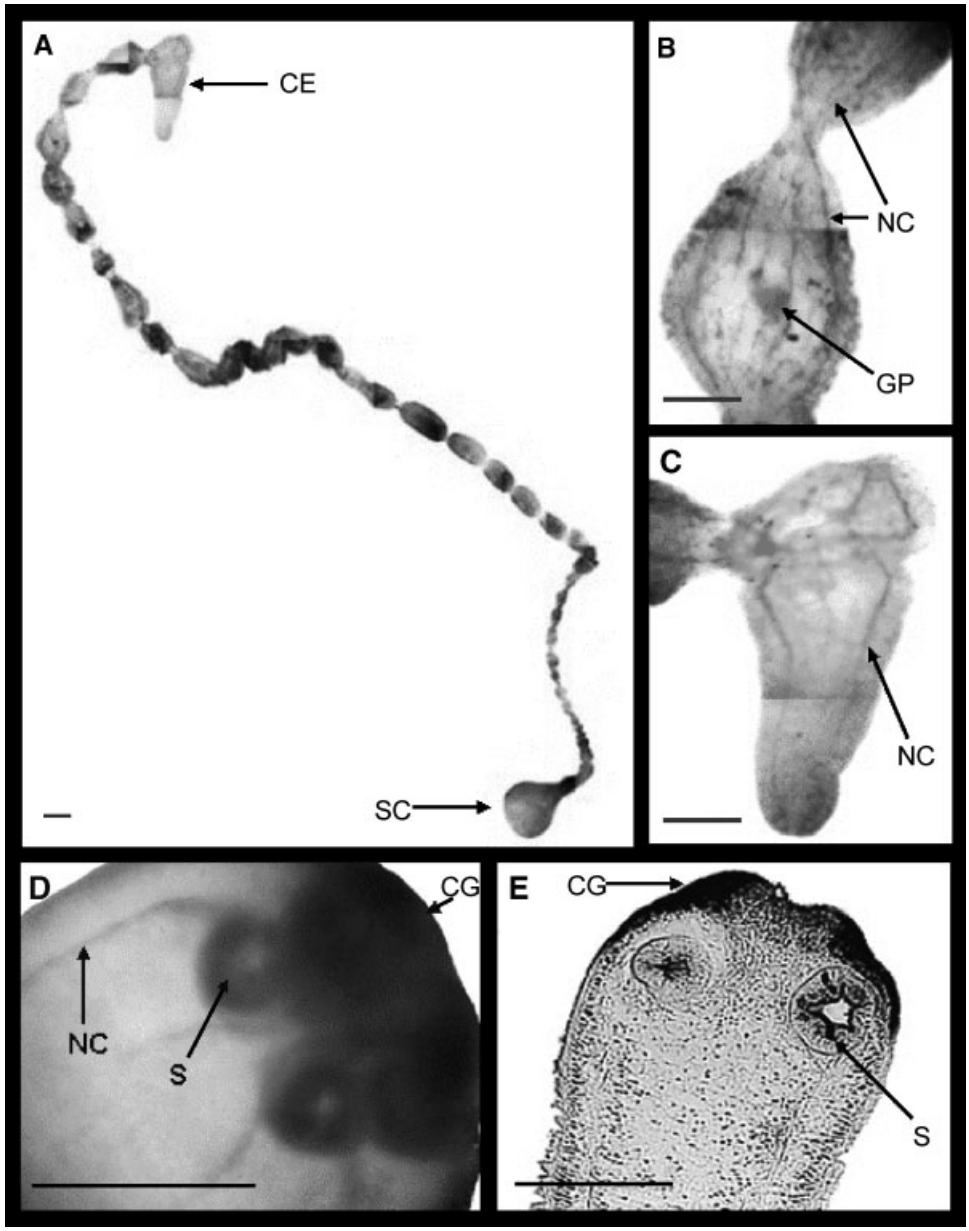

Fig. 7. AChE localization in adult worms. AChE was detected by the method of Karnovsky and Roots (1964) in whole mount preparations of adult worms (parts **A**–**D**). A histological section of the scolex stained with haematoxylin-eosin is shown in part **E**. Part A: Complete adult worm. Part B: Section showing the continuity of two proglotids corresponding to the worm shown in A; note the nerve cords passing by from one proglotid to the other. Part C: Caudal extremity of the worm shown in A; note the complexity of the nervous caudal plexus. Parts D and E: Adult scolex; note in D the strong staining of region corresponding to cerebral ganglia, shown in E. NC, nerve cords; GP, genital pore; CG, cerebral ganglia; S and SC, scolex; CE, caudal extremity. Bars: 100 μm.

the main cholinesterase activity present in the larval stage of this platyhelminth is consistent with the results obtained in free forms of other helminthes such as *Fasciola hepatica* and *Schistosoma mansoni* (Pax and Bennet, 1991), and particularly in the protoscolex (larvae) of the cestode *Echinococcus granulosus* (Giménez-Pardo et al., 2000), in which the most of cholinesterase activity corresponds to AChE. We have extended these observations to all stages of development of *M. corti* and shown that in this endoparasite AChE is the main cholinesterase activity all along the different asexual and sexual reproductive forms of the parasite.

The specific activity of AChE is relatively constant during the first stages of development of *M. corti*, increasing markedly during the transition from the segmented to the adult form of the parasite. In tetrathyridium, as well as in segmented (not shown) and adult stages of *M. corti*, the monomeric molecular form of the enzyme (G1, sedimentation coefficient 4–6 S) is present. Interestingly, in the adult worm the G1 form of AChE co-exists with a more complex molecular form, showing a sedimentation velocity (10.5 S), similar to the tetrameric (G4) AChE that was described in mouse brain (Inestrosa et al., 1994). Consequently, a rise in the AChE activity and the presence of a more complex molecular form of this enzyme is coincident with the initiation of the cellular processes leading to the adult stage of the parasite.

The change in the composition of molecular forms of AChE and the increase in its activity may reflect the presence of a more complex nervous system, necessary in the advanced stages of development of the parasite, to

adjust and co-ordinate the movement of a much bigger structure and its response to external stimuli and internal signals. This is consistent with the morphological description of the nervous system in segmented and adult worms, as compared to tetrathyridia, which also points to the presence of a more complex nervous system in developing and mature forms of the parasite. Our observations on the location of AChE in tetrathyridia are coincident with the description of Hart (1967) in the same organism. However, we were unable to appreciate five longitudinal nervous cords in this larval stage, as described by this author. This apparent disagreement may perhaps be explained by the fact that the studies of Hart were done in serial sections, while we have used only whole mount staining methods. Nevertheless, in adult worms we observed the presence of more than five longitudinal nerve cords, passing by from one proglotid to the other, corresponding to a well developed nervous system. Additionally, a marked staining for cholinesterase in the reproductive apparatus of these organisms was also observed. Platyhelminthes are characterized for an important egg production (Gustafsson, 1992) and the development of the reproductive apparatus in segmented and adult worms could explain, at least partially, the requirement of a more complex nervous system in these stages.

Our results point to AChE as a molecular marker in the development of the nervous system in platyhelminthes, as it was proposed in more complex organisms (Inestrosa et al., 1994; Moreno et al., 1998). On the other hand, AChE has also been involved in functions that are unrelated to neurotransmission but could be important in development (Small et al., 1996). Following this line of thought, the enzyme seems to be involved in processes such as cell adhesion, neuritogenesis, synaptogenesis, cell differentiation, and apoptosis (Layer, 1991; Coleman and Taylor, 1996; Small et al., 1996; Sternfeld et al., 1998; Muñoz et al., 1999; Bigbee et al., 2000; Blasina et al., 2000; Soreq and Seidman, 2001; Yang et al., 2002; Zhang et al., 2002). Considering this information, the increase in the AChE activity and the presence of a more complex molecular form of the enzyme in the adult worms of *M. corti* may be taken as an evidence of the role of this enzyme in the development of a more complex nervous system as the one needed in these forms of the parasite. It is also possible that it reflects a role of AChE in other developmental processes, such as cell proliferation and differentiation, needed for the growth of tetrathyridia to the adult worm or the maturation of the egg-producing parasite. Indeed, cell proliferation related to parasite growth was topologically found around the nerve cords in the developing stages of *M. corti* (Espinoza, 2002; Espinoza et al., 2004).

In further studies, the importance of the possible role of AChE in the development of the parasite, as well as the relationship of the enzyme with cell proliferation, cell differentiation, apoptosis, and its activity with respect to different inhibitors, would be examined. Of particular interest would be the study of the genes coding for this enzyme, as well as the levels of its messenger RNAs, in different stages of worm development. Finally, considering the availability of anticholinesterase drugs, the study of the possible morphogenetic role of this enzyme in biochemical processes occurring in the larval form, and in the cellular mechanisms related to the development and maturation of the adult worm, may be of importance for the identification of more effective therapeutic strategies against cestode parasites.

ACKNOWLEDGMENTS

Gonzalo Cabrera was supported by a fellowship from MECESUP, Chile (UCH0115).The study was supported by a grant from The Fondecyt (to N.G.)1010817, 1050135 and Network for Research and Training in Parasitic Diseases, SIDA/SAREC (to N.G) 2003/2005. We acknowledge Dr. Rodolfo Paredes for helpful discussions.

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