COMPARISON OF CHOLINESTERASE ACTIVITIES IN THE EXCRETION-SECRETION PRODUCTS OF *TRICHINELLA PSEUDOSPIRALIS* AND *TRICHINELLA SPIRALIS* MUSCLE LARVAE

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Summary :

The presence of cholinesterases (ChE) is reported in *T. pseudospiralis* excretion-secretion products (ESP) by spectrophotometric method, using acetylthiocholine (ATCI) and butyrilthiocholine (BTCI) as substrates. By inhibition assays, we found that *T. pseudospiralis* release both acetyl- and butiryl-cholinesterases (AchE and BchE, respectively). The sedimentation coefficientes of these enzymes were determined by sucrose density gradient. We studied the *in vivo* ChE secretion by immunoblot assays using AchE from *Electrophorus* (electric eel) and sera from normal or infected mice with *T. pseudospiralis or T. spiralis*. The presence of anti-AchE antibodies was only demonstrated in the sera from *T. pseudospiralis* infected mice. Moreover the *in vivo* secretion was corroborated by the high difference determinate between the ChE activity of the immuno complexes from *T. pseudospiralis* infected sera and the immunocomplexes from *T. spiralis* infected sera as well as normal sera. Finally, we analyzed the effect of the organophosphate Neguvón® (metrifonate) on the ChE activity from the *T. pseudospiralis* ESP. The drug inhibits in part this activity. Moreover Neguvón® (metrifonate) showed a high activity against the *T. pseudospiralis* viability.

KEY WORDS : *Trichinella pseudospiralis*, cholinesterase activity, excretion-secretion products.

Résumé : COMPARAISON DES ACTIVITÉS CHOLINESTÉRASIQUES DES PRODUITS D'EXCRÉTION-SÉCRÉTION DES LARVES AU STADE INTRA-MUSCULAIRE DE *TRICHINELLA PSEUDOSPIRALIS* ET *TRICHINELLA SPIRALIS*

La présence de cholinestérases (ChE) est mise en évidence au niveau des produits d'excrétion-sécrétion de T. pseudospiralis *en spectrophotométrie utilisant l'acétyl-thiocholine et la butyril-thiocholine en tant que substrats. Avec le test d'inhibition, l'on observe que* T. pseudospiralis *libère à la fois de l'acétyl- et de la butiryl-cholinestérase. Les coefficients de sédimentation de ces enzymes ont été déterminés par gradient de densité de sucrose. L'étude* in vitro *de la sécrétion de ChE a été effectuée par immunoblot utilisant de l'acétyl-cholinestérase d'*Electrophorus *(anguille électrique) et de sérums de souris saines ou infectées par* T. pseudospiralis *et* T. spiralis. *L'existence d'anticorps anti acétyl-cholinestérase n'est démontrée que dans le sérum de souris infectées par* T. pseudospiralis. *De plus,* in vivo, *la sécrétion est confirmée par la grande différence observée entre l'activité ChE des immuns complexes des sérums infectés par* T. pseudospiralis *et celle de ceux infectés ou non par* T. spiralis. *Enfin, il a été étudié l'effet du métrifonate (organophosphate) sur l'activité ChE des produits d'excrétion-sécrétion de* T. pseudospiralis. *Ce produit inhibe en partie cette activité. De plus, le métrifonate a un important effet sur la viabilité de* T. pseudospiralis.

MOTS CLÉS : Trichinella pseudospiralis, *cholinestérase, produits d'excrétion-sécrétion.*

INTRODUCTION

The presence of two types of cholinesterases (ChE), acetyl- and butyrilcholinesterases (AchE and BchE, respectively), has been recognized in crude extracts and excretion-secretion products (ESP) from different parasitic nematodes (De Vos & Dick, 1992; Massoulié *et al.*, 1993; Pritchard *et al.*, 1994). These enzymes play an important role in the nematode neuromuscular system and in the host-parasite relationship. So, secreted acetylcholinesterase (AchE) activity has been associated with the modulation of the following host mechanisms: gastrointestinal motility, cell membrane permeability, anti-coagulant processes, glycogenesis, acetate and choline metabolism, anthelmintic resistance, immune and anti-inflammatory responses (Lee, 1996). The amount of these enzymes varies from species to species, from stages to stages and between sexes, and it seems that this variation is responsible, in part, for sex-, species-, strain- and stage-specific behaviour (Lee, 1996). Several authors have analysed the ESP composition from the *Trichinella* isolates (Ko *et al.*, 1994; Modha *et al.*, 1994; Wu *et al.*, 1998; Mak *et al.*, 2000), but up to now no studies have been reported about ChE secretion by the different *Trichinella* isolates. The aim of the present report was to analyse the ChE activity from ESP of *Trichinella pseudospiralis* muscle-larvae, and to add new data about secreted molecules of this parasite and the host-parasite relationship.

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MATERIAL AND METHODS

Parasite

Muscle-stage larvae of both *T. pseudospiralis* strain (MFEL/SP/62/GM-1) (La Rosa *et al.*, 1992) and *T. spiralis* strain (MPRO/SU/72/ISSN13, T4 (Pozio *et al.*, 1989)) were obtained from NMRI mice by the standard pepsin digestion method (Brand *et al.*, 1952). These larvae were washed extensively (5x) with cell cultured medium: Hanks solution with 1 % 199 medium, (ICN FLOW), supplemented with penicillin G (1 mg/ml) and streptomycin sulphate (1 mg/ml).

Collection of ES products

Viable muscle larvae were packed under a volume of 0.5ml and were incubated at 37ºC for 24h in petri dishes, each containing 15ml of the culture medium (pH 7.2) under air with 5 % CO_2. Then the media were harvested, pooled and concentrated using a centricon-10kDa protein concentrator (ultrafree-CL PLGC, Millipore). The concentrate was dialyzed for 24h against distilled water with dialysis tubing-visking size 2-18/32"-6.3 mm 1L-14,000 daltons (Medicell International Ltd.) and used immediately or frozen at – 80ºC. The protein concentration was adjusted to 5.9 mg/ml (*T. spiralis* ESP) or 1.41 mg/ml (*T. pseudospiralis* ESP).

Preparation of the crude extract

Muscle-stage larvae from *T. pseudospiralis* and *T. spiralis* were homogenised separately in 100 mM phosphate buffer (PBS, pH7.2) and centrifuged at 100,000g for 30 minutes. The supernatants were recovered and their protein concentration was determined by the Lowry *et al.* method (1951). The protein concentration was adjusted to 4.46 mg/ml (*T. pseudospiralis*) and 8 mg/ml (*T. spiralis*).

Sucrose density centrifugation

Sucrose gradients were prepared in PBS 100 mM pH 7.5. 200µl of the samples (ESP) were loaded onto 5ml 5-20 % linear gradients and centrifuged for 18h at 4ºC in an SW 55 Ti rotor on a L8-76 ultracentrifuge at 4,700rpm. ß-galactosidase (16S), catalase (11.3S) and alkaline phosphatase (6.1S) were included as standards and sedimentation coefficients were calculated as described by Martin & Ames (1961).

Enzyme assays

Cholinesterase activity

Cholinesterase activity was determined by a modification of Ellman's method (Rathaur *et al.*, 1987) using a microplate in which 260µl of 100 mM PBS buffer pH 8, 10µl of 10 mM DTNB (39.6 mg dithiobisnitrobenzoic acid solution, 10ml PBS 100 mM pH 7, 15 mg sodic bicarbonate), 2.5µl of 75 mM acetilthiocholine iodide (ATCI) or butyrylthiocholine iodide (BTCI) and 25µl of the sample (1 mg/ml) were incubated. The O.D. increment was measured every min during a 10 min interval at 412nm.

β-galactosidase activity

ß-galactosidase activity was measured by the change in O.D. at 410nm of 200µl assay solution (0.1 M PBS pH 7.5, 2 mM 0-nitrophenyl-α -D-galactopyranoside, 10 mM NaCl, 1 mM $MgCl_2$ and 0.1 M ß-mercaptoethanol) which was added to 10µl of the sample in a microplate.

Alkaline phosphatase activity

Alkaline phosphatase activity was measured by the change in O.D. at 410nm in microtitre plates in which 200µl of substrate (0.1 M Tris HCl pH 8.5, 1 mM sodium-p-nitrophenyl phosphate) was added to 10µl of the sample.

Catalase activity

Catalase activity was determined by measuring the change in O.D. at 240nm every min during the 5 min period in the 1ml assay solution (0.05 M PBS buffer pH 7, 0.02% H_2O_2) containing 10µl of the sample.

Inhibition assays

9µl of the inhibitors 1.5-bis-(4 allyldimethylammoniumphenyl-)-pentan-3-one (BW284C51), tetraisopropylphosphoramide (isoOmpa) or the metrifonate (ICD) at various concentrations were incubated with 16µl of the samples at a final protein concentration of 1 mg/ml during 10 min at room temperature, and ChE activity was measured by the Ellman method modified by Rathaur *et al.* (1987) as explained above. Inhibition was calculated as the percentage of the activity of positive controls, as measured in the microassay.

Isolation of immune complexes

The immune complexes from the sera were isolated as previously described Weil & Liftis (1987) by overnight incubation at 4ºC in PBS 100 mM pH 7.2 suplemented with a 8 % PEG 4000 (polyethylenglycol 4000, Merck). Then, precipitates were washed three times with 4 % PEG in PBS 100 mm pH 7.2 and measured for ChE activity by the Ellman method modified by Rathaur *et al.* (1987).

WESTERN BLOTTING

8µg of *Electrophorus* (electric eel) purified AchE (SIGMA) were electrophoresed on a 12 % SDS-PAGE. After the gel was equilibrated in Western-blot buffer (25 mM Tris, 192 mM glycin, 20 % methanol pH 8.3, for 10 min.) and transferred onto nitrocellulose membrane. Transfer was carried out during 90 min at 153 mA and 4ºC. Non-specific binding sites on the membrane were blocked with 10 % skimmed milk in PBS for 1h at 25ºC. Then the blots were washed three times in PBS supplemented with 0.05 % Tween 20 (PBS-T20) and then incubated overnight at 4ºC with sera from normal or infected (by *T. spiralis* or *T. pseudospiralis*) mice, diluted 1:20 in PBS-T20. Three washings were then followed by incubation for 1h with goat anti-mouse IgG, conjugated to peroxidase (SIGMA), diluted 1:1,000 in PBS-T20. The blots were washed three times in PBS-T20 before incubation with the substrate (3, 3 diamino-benzidine, SIGMA; diluted in PBS with 0.05 % (v/v) H_2O_2).

ANTHELMINTIC ASSAYS

The anthelmintic activity of metrifonate (ICD) was studied in *T. spiralis* and *T. pseudospiralis* (adult worms, L_1 migrating and muscle larvae). The following experimental protocol was applied. NMRI mice were orally infected with 300 ± 50 larvae/mice of *T. spiralis* or *T. pseudospiralis*, respectively, and were treated per os with a single dose of metrifonate (ICD) (100 mg/kg) at day 3, 15 or 40 p.i. against adults, migrating and muscle larvae respectively and the animals were killed at day 6, 30 and 50 p.i., respectively. The recovery of the adult worms and muscle larvae (30 and 50 days p.i.) was carried outly the methods of Denham & Martinez (1970) or Brand *et al.* (1952).

CHEMICALS

ATCI, BTCI, AchE, BW284C51, isoOMPA, ß-galactosidase, catalase, alkaline phosphatase and PEG 4000 were purchased from SIGMA. All other chemicals were of analytical grade. Electrophoresis calibration kits were obtained from Bio-Rad.
Metrifonate was purchased from BAYER.

RESULTS

This work has revealed an important fact, the ChE excretion-secretion by *T. pseudospiralis* and *T. spiralis* muscle larvae and no by *T. spiralis* larvae. These enzymes seems to be AchE and BchE, so we determine activity against both ATCI and BTCI (Table I). Moreover, we determined that the activity of *T. pseudospiralis* crude extract is two fold higher than that of *T. spiralis* against both substrates, although BTCI degradation was 50 % less by both parasites.

ChE Activity (U/mg prot) (*)

Sample	ATCI	BTCI
T. pseudospiralis (CE)	1.9352 ± 0.870	1.0173 ± 0.164
T. pseudospiralis (ESP)	0.3188 ± 0.156	0.040 ± 0.007
T. spiralis (CE)	0.9565 ± 0.0079	0.5384 ± 0.006
T. spiralis (ESP)	0	0

CE = Crude extract
ESP = Excretion-secretion
SE = Standard error
(*) = One unit is a 1mmol of substrate hydrolized per minute per mg of protein at 20ºC. The data represent the mean of six experiments ± standard error.

Table I. – ChE activity in crude extracts and ESP of *Trichinella pseudospiralis* and *T. spiralis* using ATCI or BTCI as substrates.

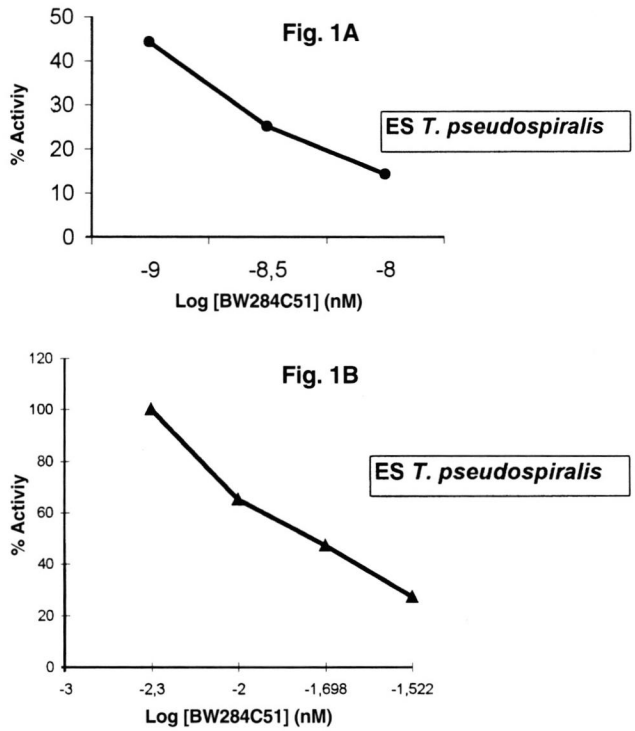

Fig. 1. – Inhibition profiles of ChE activity of ES products from *T. pseudospiralis* larvae by the AchE inhibitor BWC284C51 (nM) (A) and BchE inhibitor isoOMPA (B) (mM) when the substrate used was ATCI.

The use of specific inhibitors BW284C51 and isoOMPA employing ATCI as substrate have demonstrated that ChE of the *T. pseudospiralis* ESP are AchE and BchE, so both inhibitors decreased the ChE activity, as shown in the Fig. 1, being BW284C51 Ic_{50} 1nM and isoOMPA Ic_{50} 20 mM (Ic50 is defined as the inhibitor concentration necessary to decrease a 50 % the enzyme activity).

The enzyme characterization by sucrose density centrifugation revealed that the ChE released by *T. pseu-*

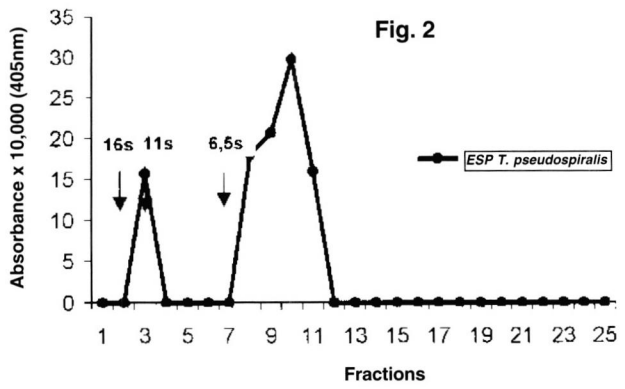

Fig. 2. – Sucrose density gradient profiles for ESP of *T. pseudospiralis*. Che activity was measured using ATCI as substrate and it is presented as the change in optical density at 412nm for 25µl aliquots of 200µl fractions from 5-20% sucrose gradients. Sedimentation coefficients were determined by comparison with standards: b-galactosidase (16S), catalase (11S) and alkaline phosphatase (6.1S).

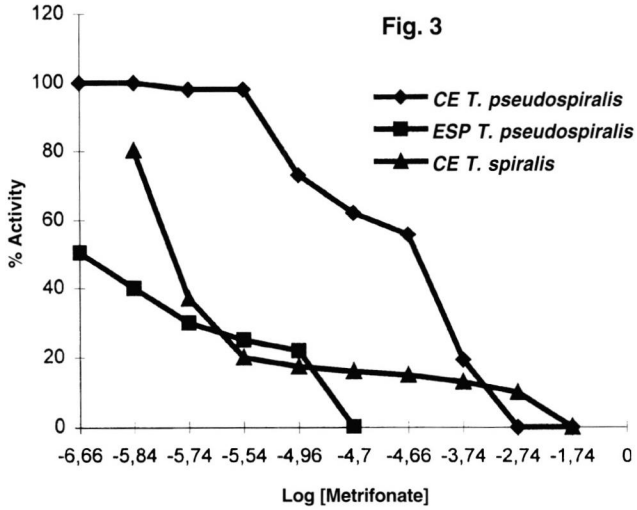

Fig. 3. – Inhibition profiles of ChE activity of crude extracts (CE) of *T. spiralis* and *T. pseudospiralis* and excretion-secretion products (ESP) from *T. pseudospiralis* by metrifonate when the substrate used was ATCI.

dospiralis are separated in two peaks of activity at 11.5S and 2.5S (Fig. 2).

When metrifonate was employed, an inhibitory effect on ChE activities from crude extracts and ESP from *T. pseudospiralis* and *T. spiralis* using ATCI as substrate was observed (Fig. 3). Note the highest inhibitor effect on *T. pseudospiralis* ESP enzymes (Ic_{50} = 0.00026 mM) followed by *T. spiralis* crude extract (Ic_{50} = 0.00036 mM) and finally on *T. pseudospiralis* crude extract (Ic_{50} = 0.063 mM).

The "in vivo" secretion of some of these enzymes by *T. pseudospiralis*, was confirmed using two different methods. First, we analyzed the presence of specific anti AchE antibodies by immunoblot assays using *Electrophorus* (electric eel) AchE. Then, we compared the AchE activity of the immuno complexes from normal and infected mice (*T. spiralis* and *T. pseudospiralis*) as well as before and after oral administration of a single dose of metrifonate.

Immunoblot assays using AchE from *Electrophorus* (electric eel) demonstrated the presence of circulating antiChE antibodies only in the sera from *T. pseudospiralis* infected mice, while there were no antibodies in the *T. spiralis* infected mice and normal serum samples (Fig. 4 B, C, D). Moreover, the immuno complexes from the serum of *T. pseudospiralis* infected mice had more than twice the ChE activity of the normal serum (Table II). No difference was determined between ChE activities of the immuno complexes from *T. spiralis* infected mice (6, 30 or 40 days p.i.) and normal mice. On the other hand, ChE activity of the immuno complexes from *T. pseudospiralis* infected mice treated with metrifonate was lower than the activity of the immuno complexes from *T. pseu-*

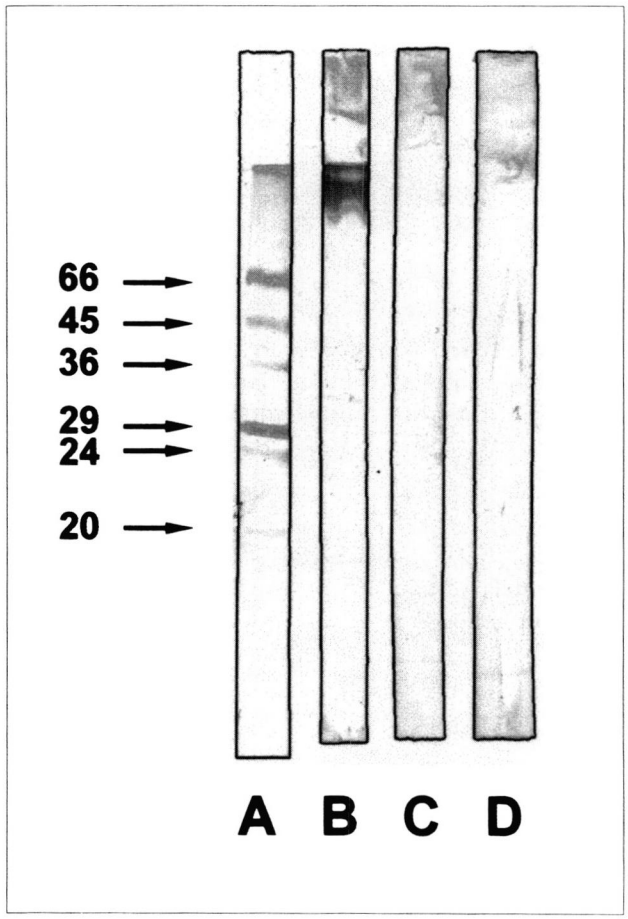

Fig. 4. – Western-blot analysis indicating the "in vivo" AchE secretion of *T. pseudospiralis* muscle-larvae. Following SDS-PAGE of the ChE from *Electrophorus* (electric eel), blots were probed with normal mice sera (C), and sera from infected mice with 300±50 larvae/mice of *T. spiralis* (D) or *T. pseudospiralis* (B). Molecular-weight markers are shown on the figure (A).

Serum	ChE activity (U/ml serum) (+)					
	Adults (1)		30 days p.i. (2)		40 days p.i. (3)	
N = sample size	Control	Treated	Control	Treated	Control	Treated
Non infected (N = 10)	0.742 ± 0.0007	0.760 ± 0.007	0.742 ± 0.0007	0.759 ± 0.0007	0.742 ± 0.0007	0.749 ± 0.0007
Infected with *T. spiralis* (N = 10)	0.7715 ± 0.05	0.6832 ± 0.0119	0.6305 ± 0.0007	0.5974 ± 0.0269	0.6461 ± 0.0496	0.64 ± 0.05
Infected with *T. pseudospiralis* (N = 10)	3.038 ± 0.042	0.5742 ± 0.241*	1.57 ± 0.011	0.357 ± 0.054*	1.8013 ± 0.037	1.2212 ± 0.027*

* $p < 0.05$
(1): metrifonate doses: 100 mg/kg at day 3 p.i. Mice sacrificated at day 6 p.i.
(2): metrifonate doses: 100 mg/Kg at day 15 p.i. Mice sacrificated at day 30 p.i.
(3): metrifonate doses: 100 mg/Kg at day 30 p.i. Mice sacrificated at day 40 p.i.
U: 1 unit is expressed in µmol of substrated hydrolized per minute.
+: The data represent the mean of the ChE activity of immunocomplexes from the sera of 10 mice ± standard error.

Table II. – ChE activity of the PEG precipitates from normal and infected mice sera (300 ± 50 larvae/mouse of *T. pseudospiralis* or *T. spiralis* respectively), before (control) and after (treated) the oral administration of a single dose of metrifonate. The substrate used was ATCI.

	Sample			
	T. spiralis		***T. pseudospiralis***	
	Control	Treated	Control	Treated
(1) Nº adults % reduction	135.8 ± 33.6	77.6 ± 11.43* 42.8 %	127 ± 20.62	17.5 ± 5.26* 85.82 %
(2) Nº larvae % reduction	20397 ± 1195	20189.7 ± 737 (NS) 1 %	2274.95 ± 275	1691.625 ± 72* 25.64 %
(3) Nº larvae % reduction	13922.22 ± 560	11783.33 ± 854 (NS) 15.36 %	12541.655 ± 572	5220.85 ± 252* 58.37 %

*$p < 0.05$
NS: Not significative
(1): metrifonate doses: 100 mg/Kg administered at day 3 p.i. Mice sacrificated at day 6 p.i.
(2): metrifonate doses: 100 mg/Kg administered at day 15 p.i. Mice sacrificated at day 30 p.i.
(3): metrifonate doses: 100 mg/kg administered at day 30 p.i. Mice sacrificated at day 40 p.i.

Table III. – Number of adults or larvae recovered from the mice inoculated with *T. pseudospiralis* or *T. spiralis* (300 ± 50 larvae/mouse) before (control) and after (treated) the oral administration of a single dose of metrifonate.

dospiralis infected mice and no treated with the drug. Never the serum ChE activities values were significatively modified by treatment with metrifonate respect their control sera from untreated mice.

Finally, we determined the efficacy of metrifonate against adults, newborn and muscle larvae. Results are shown in Table III. Metrifonate reduces the number of *T. spiralis* adults by 42.8 % but it is not effective against the larvae. However, metrifonate is effective against different stages of *T. pseudospiralis*, reducing by 85 % (adults), 25 % migrating L_1 larvae and 58 % (muscle larvae), the number of worms ($p < 0.05$).

DISCUSSION

It is known that ChE secretion shows quantitative and qualitative differences between species and that these differences could be the cause of different host-parasite interrelationships (Lee, 1996). On the other hand, the various ESP composition of *T. spiralis* and *T. pseudospiralis* muscle-larvae is known as well as their different behaviour in the muscles (Ko *et al.*, 1994; Wu *et al.*, 1998; Mak *et al.*, 2000). The present report constitutes the first study about a variability between *in vivo* and *in vitro* ChE secretion of two *Trichinella* isolates, *T. spiralis* and *T. pseudospiralis*. Moreover, we have demonstrated the efficacy of an organophosphate, metrifonate, an anti-AchE drug, against the various stages of *Trichinella*. Previously, Martinez Fernandez *et al.* (1967) observed the inefficacy of several organophosphates against *T. spiralis* muscle larvae but no results have been reported with *T. pseudospiralis*.

Through spectrophotometrical assays, we demonstrate ChE activity in crude extracts of both *T. spiralis* and *T. pseudospiralis*, though this activity is two fold higher in the case of *T. pseudospiralis* against both substrates

ATCI and BTCI. On the other hand we determined *T. pseudospiralis* is the only one who excretes-secretes ChE to the medium and some of these enzymes are recognized by the host immune response as occurs with other nematodes (Pritchard *et al.*, 1994) and may produce the antibodies synthesis as we demonstrate by Western-blot and immunocomplexes assays.

The secretion of these enzymes and other known molecules (Ko *et al.*, 1994; Wu *et al.*, 1998; Mak *et al.*, 2000) could be involved in the two different host-relationship of *T. spiralis* and *T. pseudospiralis* as ocurs with other parasites (Lee, 1996). Moreover, it seems that the secretion of these enzymes is essential for the parasite life because its inhibition by metrifonate causes the parasite death. So, while this drug is effective only on *T. spiralis* adults, the only ones that excrete-secrete ChE (data in press), this drug is effective against every studied *T. pseudospiralis* stages.

In summary, i) *T. pseudospiralis* released ChE during its complete life cycle; ii) It seems that this excretion-secretion is vital, so its inhibition by metrifonate decreases significantly the *T. pseudospiralis* survival and iii) the ChE released produces a specific immune response that provides a difference between parasitized mice and non-infected ones. For all these reasons we conclude that the identification of AchE released by *T. pseudospiralis* constitutes a great approach for the explication of the different behaviours of the *Trichinella* isolates, although further *in vivo* studies using inhibitors and stimulation of this activity are necessary to confirm this fact. Moreover, AchE by its capacity to stimulate a specific immune response is a potential antigen useful for *T. pseudospiralis* infection immuno diagnosis and/or immuno prophylaxis, as has been proposed for other parasite released AchE (Lee, 1996; Rathaur *et al.*, 1992).

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We declare that all the experiments comply with the current laws of Spain where the experiments were performed.

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