

TRANSGLUTAMINASE ACTIVITY IN EQUINE STRONGYLES AND ITS POTENTIAL ROLE IN GROWTH AND DEVELOPMENT

RAO U.R.***, CHAPMAN M.R.*, SINGH R.N.***, MEHTA K.*** & KLEI T.R.*, **

Summary :

Transglutaminases (E.C. 2.3.3.13) are a family of Ca²⁺-dependent enzymes that stabilize protein structure by catalyzing the formation of isopeptide bonds. A novel form of transglutaminase has been identified and characterized that seem to play an important role in growth, development, and molting in adult and larval stages of filarial nematodes. The aim of this study was to identify the ubiquitous nature of this enzyme in other nematodes and to measure its significance to larval growth, molting, and development. For this purpose, equine *Strongylus* spp. were used. Activity of this enzyme was identified in extracts of larvae and adults of *Strongylus vulgaris*, *S. edentatus*, *Parascaris equorum* and *Cylicocyclus insigne*. The significance of transglutaminase in the early growth and development of *Strongylus vulgaris*, *S. edentatus* and *S. equinus* was tested by adding specific inhibitors, monodansylcadaverine (MDC) or cystamine (CS), to *in vitro* cultures of third (L3) and fourth stage larvae (L4). The viability, molting and growth of these nematode species were affected by both inhibitors. Cystamine promoted abnormal development of *Strongylus edentatus* L3, resulting in an aberrant expansion of the anterior end. Addition of these inhibitors to cultures of L4 also reduced growth of the three species. The results indicated that transglutaminase is present in a wide array of nematode parasites and may be important in growth and development of their larval stages.

KEY WORDS : equine, strongyles, L3, L4, transglutaminase, growth, molting, inhibitors, monodansylcadaverine (MDC), cystamine (CS).

Résumé : ACTIVITÉ ET RÔLE POTENTIEL DE LA TRANSGLUTAMINASE DANS LA CROISSANCE ET LE DÉVELOPPEMENT DES STRONGYLES ÉQUINS

Les transglutaminases (E.C.2.3.3.13) sont une famille d'enzymes Ca²⁺-dépendantes qui stabilisent la structure protéique en catalysant la formation de ponts isopeptidiques. Une forme nouvelle de transglutaminase a été identifiée et caractérisée; elle semble jouer un rôle important dans la croissance, le développement et la mue des stades adultes et larvaires des filaires. L'objectif de cette étude était d'identifier la nature ubiquitaire de cette enzyme chez d'autres nématodes et d'en mesurer l'impact sur la croissance, la mue et le développement larvaires.

Dans ce but, des espèces de *Strongylus* équines ont été utilisées. L'activité enzymatique a été identifiée à partir d'extraits de larves et d'adultes de *Strongylus vulgaris*, *S. edentatus*, *Parascaris equorum* et *Cylicocyclus insigne*. L'impact de la transglutaminase dans la croissance précoce et le développement de *Strongylus vulgaris*, *S. edentatus* et *S. equinus* a été testé par l'addition d'inhibiteurs spécifiques, monodansylcadaverine (MDC) ou cystamine (CS) aux cultures *in vitro* de larves de troisième (L3) et de quatrième stade (L4). La viabilité, la mue et la croissance de ces espèces de nématodes ont été affectées par les deux inhibiteurs. La cystamine a entraîné un développement anormal des L3 de *Strongylus edentatus*, se traduisant par une expansion aberrante de l'extrémité antérieure. L'addition de ces inhibiteurs aux cultures de L4 a réduit la croissance des trois espèces. Ces résultats indiquent que la transglutaminase est présente dans toute une collection de nématodes et peut être importante pour la croissance et le développement de leurs stades larvaires.

MOTS CLÉS : équin, strongyles, L3, L4, transglutaminase, croissance, mue, inhibiteur, monodansylcadaverine (MDC), cystamine (CS).

INTRODUCTION

The highly complex nematode cuticles have been considered as collagenous and crosslinked by strong disulfide bridges (Cox *et al.*, 1981; Selkirk *et al.*, 1989; Maizels *et al.*, 1993; Geary *et al.*, 1995). It

is interesting that the external cortical layer and the epicuticle are highly insoluble proteins covalently cross-linked by nonreducible bonds (Fetterer, 1989; Cox, 1992; Maizels *et al.*, 1993). During the development of cuticulin, such cross linking has been shown previously. Dityrosine and isotryrosine in cross linking of cuticular proteins of nematodes has been extensively studied, yet the mechanism of synthesis of these crosslinks is unknown (Fujimoto *et al.*, 1981; Fetterer & Rhoads, 1990; Fetterer & Hill, 1993; Fetterer *et al.*, 1993). In addition, activity of phenol oxidase in protein cross linking and egg shell development of *Trichuris* has been suggested (Fetterer & Hill, 1993). Filarial nematode cuticle development and sheath formation have been attributed to crosslinking of amino acids, resulting in a highly insoluble fraction of the somatic proteins (Selkirk *et al.*, 1989;

* Department of Veterinary Science,

** Department of Microbiology and Parasitology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana,

*** Department of Bioimmunotherapy, The University of Texas M.D. Anderson Cancer Center, Houston, Texas.

Correspondence: U.R. Rao. Ph.D., Washington University School of Medicine, Infectious Diseases Division, Barnes Jewish Hospital (N), 216 South Kings Highway, St.Louis, MO 63110.

Tel.: 314 454 7483 – Fax: 314 454 5293.

Email: RRAO@imgate.wustl.edu

Hirzmann Jorg *et al.*, 1995; Zahner *et al.*, 1995). Similarly, the highly insoluble complexes of larval or adult strongyle extracts reveal the presence of similar cross linking of isopeptide bonds. Recently, transglutaminase (TGase), a new type of protein cross linking enzyme, has been observed in lymphatic filarial nematodes (Mehta *et al.*, 1990, 1992; Rao *et al.*, 1991), as well as *Dirofilaria immitis* (Singh *et al.*, 1995) and *Onchocerca volvulus* (Lustigman *et al.*, 1995), and possibly plays a role in molting, growth, and development of these parasites. Moreover, TGase was also involved in covalent incorporation of host proteins into developing stages of *Brugia malayi* (Mehta *et al.*, 1996).

Equine strongyles are pathogenic gastrointestinal nematodes of horses and have been associated with a wide range of clinical syndromes (DiPietro *et al.*, 1990; Klei, 1992). In immune and non-immune equines, the infection is always transmitted by ingested third stage larvae (L3). The three *Strongylus* species that we have used in this study have complex life cycles and each of them are different. These larvae exsheath in the intestine and migrate through the mucosa. Exsheathed L3 of *S.edentatus* migrate through the venous system to the liver during the first 40 hrs of the infection and eventually to the colon (McCraw & Slocombe, 1974). L3 of *S.equinus* migrate into the liver via the peritoneal cavity, and then to the colon via pancreas (McCraw & Slocombe, 1985). However, molting, growth, and development through L4 and L5 stages of *S.vulgaris* occur in the arterioles (Enigk, 1970). Pathological symptoms are usually associated with these early developmental stages during their tissue migration (Drudge *et al.*, 1966, 1989; Enigk, 1970; McCraw & Slocombe, 1985). Although the periodic treatment by anthelmintics, e.g., pyrantel pamoate, benzimidazoles and ivermectin, is highly efficacious against migrating strongyle larvae; the latter two are active against larvae and adults. Therefore, a specific and targeted approach in designing new compounds has always been a better choice in new drug development. This approach is worth pursuing as there has been emerging evidence of drug resistance in strongyles (Slocombe, 1992). The objective of the present study was to investigate the presence of transglutaminase activity in different species of strongyle nematodes and to measure its biological activity in molting, growth, and development of L3 and L4 stage larvae *in vitro* by using two known transglutaminase inhibitors, monodansylcadaverine and cystamine.

MATERIALS AND METHODS

PARASITES

Strongylus vulgaris L3 and *S. edentatus* L3 were obtained from ponies with surgical implantation of monospecific infections (McClure *et al.*, 1994).

Strongylus equinus L3 were recovered from naturally infected ponies. Adult male and female worms of these species recovered from intestines were washed repeatedly in phosphate-buffered saline (PBS). Developing larvae and adults of *Parascaris equorum* and *Cylicocyclus insigne* were also collected from naturally infected ponies at necropsies and washed thoroughly in PBS. All these adult worms or L3 stages were stored in PBS at -70 C until use.

L3 recovery from fecal collection, culture, purification and further exsheathing were essentially carried out as described by Farrar & Klei., (1985) and Klei *et al.*, (1982). Larvae were cultured using media and methods as described by Chapman *et al.*, (1994) to obtain L4 stages. For soluble parasite extract preparations, L3 (ensheathed), L3 (exsheathed), L4, and male and female *S. edentatus* were homogenized separately in Tris-HCl buffer (20 mM), pH 8.5, containing a cocktail of 2 mM 1,4-dithiothreitol, 1 mM ethylenediamine tetra acetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-tosyl-L-lysine chloromethane, 0.1 mM N-tosyl-L-lysine chloromethane, and 0.1 mM N-tosyl-L-phenylalanine chloromethane for 20 min on ice. The homogenized extract was then sonicated continuously for three cycles of one min, with five min stop-cycle, using a W-225 ultrasonic processor on ice. The crude extract was mixed with 0.1 % Triton x-100 and resonicated for five min. The suspension was centrifuged at 15,000 g for 20 min; supernatant was filtered through a sterile millipore filter (0.2 µm). Protein concentrations of soluble extracts were estimated with a commercial kit (Pierce Chemical Co., Rockford, Illinois) and stored at -70 C until use.

INHIBITORS

Two TGase inhibitors were used, e.g., monodansylcadaverine (MDC), a competitive substrate inhibitor; and cystamine (CS), an active-site inhibitor (Sigma chemical company, St. Louis, Missouri). Millimolar concentrations of these inhibitors were prepared before each experiment and the pH of the culture solution was adjusted if necessary after the addition of the inhibitor such that it was maintained at 7.2-7.5.

IN VITRO CULTURE OF L3 AND L4

Culture of equine strongyles from third to the fourth stage larvae using standard medium and gas phase (10 % CO₂, 5 % O₂ and 85 % N₂) always yielded 44-95 % molting depending on the species (Chapman *et al.*, 1994). Under these conditions, L3 were cultured *in vitro* for 6-10 days depending on species. TGase inhibitors were added to these cultures at day 0, 6, or 11 at increasing concentrations from 0.05, 0.1, 0.3,

0.5 and 1 mM. The number of molting larvae was determined on day 4 and 6 for *S. vulgaris*, and day 6 and 10 for *S. edentatus* and *S. equinus*. Larval viability was measured by microscopic observation of motility and integrity. Duplicate samples of the cultures were obtained at each time to measure the growth and development. For this purpose, larval stages were transferred to clean glass slides. The width and length of these stages were scored morphometrically using a compound microscope fixed with a ocular micrometer. When larval movement was sufficient to interfere with measurements, a drop of 2 % NaAzide was added to the side of the coverslip to paralyze the larvae. Larval samples were prepared in a similar manner to determine the abnormal changes during larval development. Experiments were repeated and the results on viability, molting, and growth for each inhibitor concentration were shown as an increase or decrease in percent when compared with those of control larvae cultured in parallel without inhibitors.

TGASE ACTIVITY IN SOLUBLE EXTRACT PREPARATIONS

Enzyme activity in soluble extracts of L3, L4, and males and females was determined according to the previously described method (Singh & Mehta, 1994). In brief, microtiter plates were coated with 0.2 % dimethyl casein at 37 C for 1 hr. The wells were blocked with 0.5 % nonfat dry milk; 200 μ l of reaction mixture containing 100 mM Tris-HCl (pH 8.5), 10 mM CaCl₂ or ethylenediaminetetraacetic acid (EDTA), 10 mM dithiotheritol (DTT), 1 mM BPA (5[biotinamido]pentylamine) and the soluble extract was added to each well and incubated at 37 C for 1 hr. Enzyme catalyzed conjugation of BPA into dimethyl casein was detected by streptavidin-alkaline phosphatase and p-nitrophenyl phosphate as a substrate; 2 mM of sodium bicarbonate was used as stopping solution of the enzyme reaction, and absorbance was determined at 405 nm using a Vmax kinetic microplate reader (Molecular Devices, Palo Alto, California). Known concentrations of guinea pig liver transglutaminase (GPTGase) was used in each plate to generate a standard curve under similar conditions.

STATISTICAL ANALYSIS

Significance of differences in larval molting and growth was tested by using 1-way analysis-of- variance; all pairwise multiple comparisons were made using the Student-Newman-Keuls method, or Dunn's method, or both. The differences between the groups were considered significant when the *P* values were < 0.05.

RESULTS

TGASE ACTIVITY IN SOLUBLE EXTRACTS OF LIFE CYCLE STAGES OF *STRONGYLUS EDENTATUS*, *PARASCARIS EQUORUM* AND *CYLICOCYCLUS INSIGNE*

Strongylus edentatus, due to its abundance, was used as the base-line species to measure the activity of TGase *in vitro* by employing a highly sensitive microwell plate assay that utilizes a chemical reaction between an amine acceptor (N-N-dimethylcasein) and an amine donor substrate BPA (Singh & Mehta, 1994). High levels of TGase were detected by this method, e.g., 160 ng/mg/protein (in L3 at day 3 of culture) and 100 ng/mg/protein (in L4 at 22 days of culture). The levels were insignificant in larvae collected from cultures where molting did not occur. TGase was 262 and 205 ng/mg protein in developing male and female worms at the L5 stages, respectively. Their concentrations were higher in mature adult males (214 ng/mg/protein) than mature females (158 ng/mg/protein). It is interesting that the enzyme activity is Ca⁺²-dependent in this assay, an important feature of TGase catalyzed reactions, and could be inhibited by chelating agent EGTA (data not shown). The enzyme activity was also observed in soluble extracts of young adults of *Parascaris equorum* (130.5 ng/mg/protein) and in developing L3 (87 ng/mg/protein) and adults (360 ng/mg/protein) of *Cylicocycclus insigne*.

EFFECT OF TGASE INHIBITORS ON L3 VIABILITY, MOLTING AND GROWTH

In an initial experiment to determine whether TGase catalyzed reactions have any role in the viability of *Strongylus* spp., the effect of two known TGase inhibitors was tested on the viability of *S. edentatus*, *S. vulgaris*, and *S. equinus* L3. Cystamine, an active site inhibitor, or MDC, a potent competitive substrate inhibitor that can be assimilated and is effective in blocking the endogenous TGase catalyzed protein crosslinking, were added to the L3 cultures at mM concentrations. The viability of larvae in these cultures was determined microscopically during the culture period. Cystamine at 0.5 mM showed no effect on viability of any of the three species (Figs. 1A-C). However, at an equimolar concentration, MDC induced significant reduction in larval viability of *S. edentatus* and *S. equinus*, but not *S. vulgaris*. CS did not affect the larval viability at 1 mM, whereas, in contrast MDC induced a marked inhibitory effect on larval viability of all three strongyle species (data not shown). These results suggest that the uptake or absorption rate of these inhibitors or their mode of action may not be equal within these species, thus producing the differences seen in viability at posttreatment times.

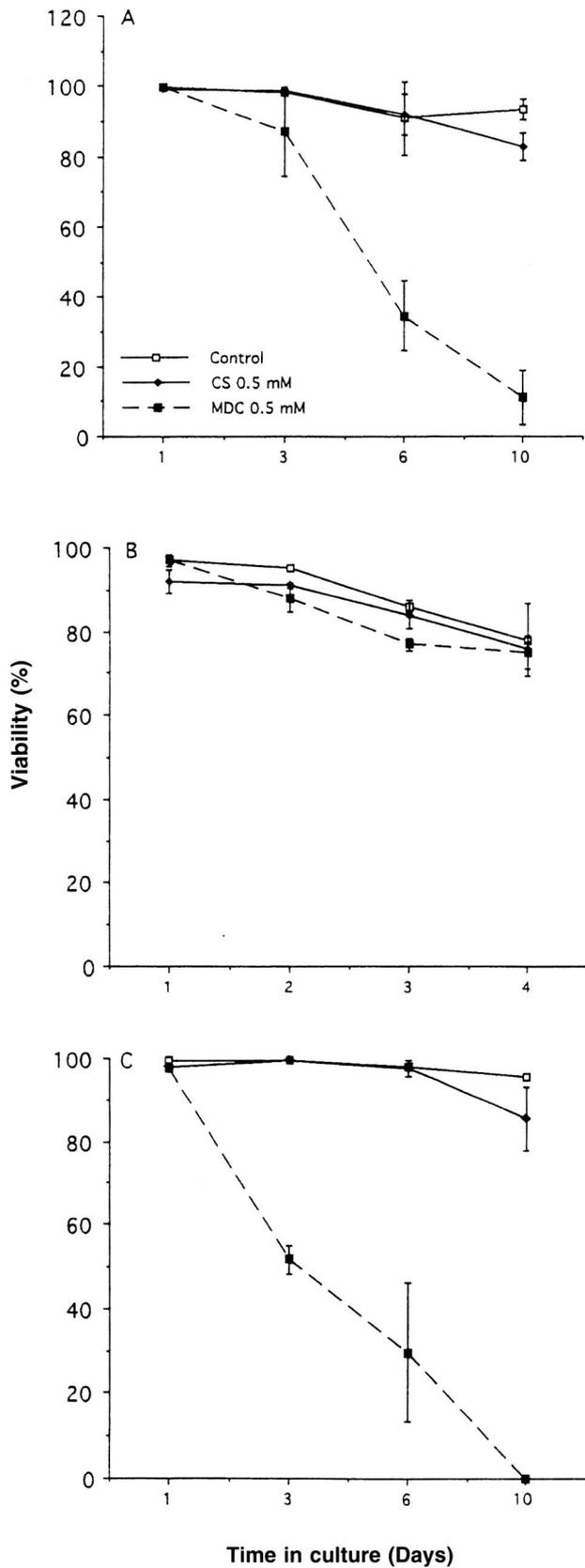


Fig. 1. - *In vitro* effect of MDC and CS on *Strongylus edentatus* (A), *S. vulgaris* (B) and *S. equinus* (C) L3 viability. Results are expressed as means \pm SE of duplicate samples.

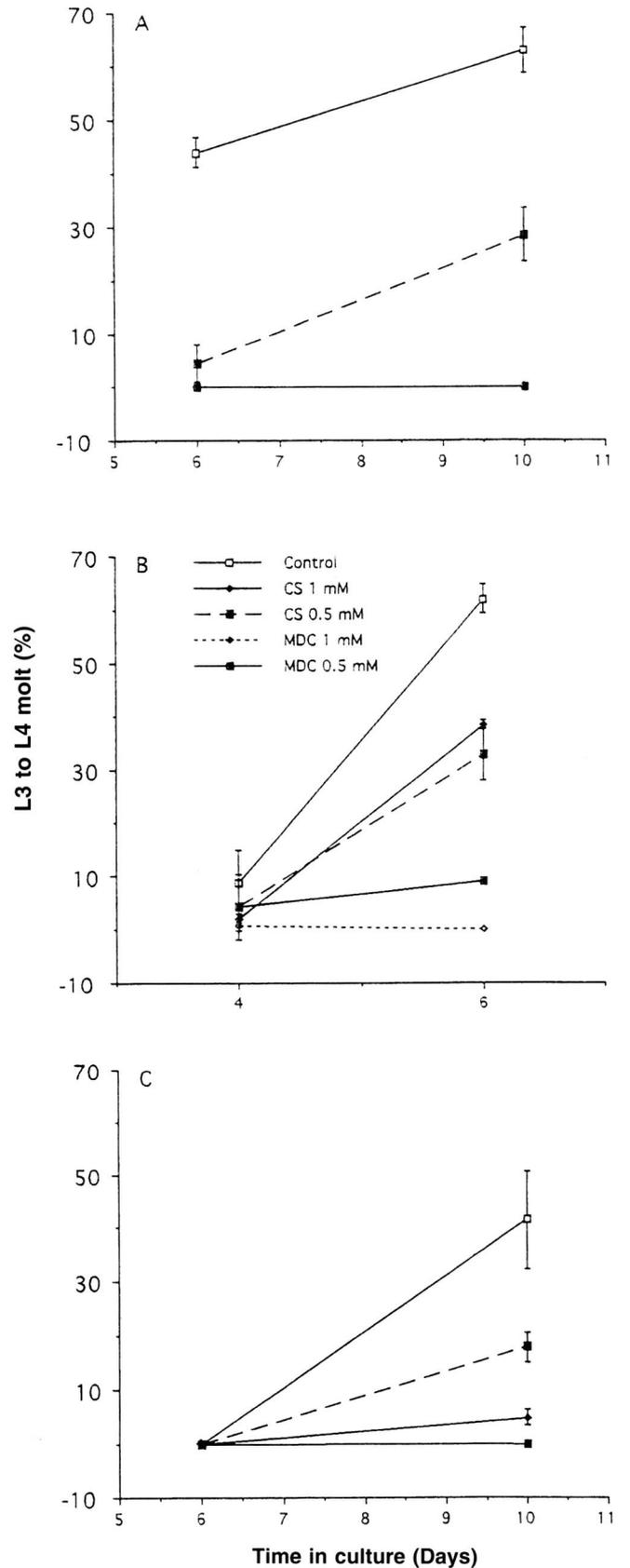


Fig. 2. - *In vitro* effect of MDC and CS on *Strongylus edentatus* (A), *S. vulgaris* (B) and *S. equinus* (C) L3 molting. Results represent means \pm SE of duplicate samples.

The effect of these two inhibitors on molting of L3 to L4 was also tested. Culture periods selected for molting and viability measurements of each species were based on previous observations (Chapman *et al.*, 1994). To measure the effect of inhibitors, larval cultures were supplemented with two concentrations of CS or MDC and incubated as previously described. Cystamine at 1 mM and MDC at both the concentrations tested, greatly inhibited *S. edentatus* L3 molting to L4 when compared with controls (Fig. 2A). Cystamine at 0.5 mM was marginally effective compared to that at 1mM. Molting was completely inhibited by day 10 in cultures containing CS at 1mM; and MDC at 0.5 or 1mM. Similarly, Figure 2B shows the effect of both MDC and CS on *S. vulgaris* L3 molting to L4. Both the inhibitors reduced the molting by day 6. However, the effect of MDC was much greater than CS at these concentrations. Whereas MDC at 0.5 mM and 1mM caused 80-100 % inhibition in *S. vulgaris* molting to L4, CS reduced molting by 37 and 46 % on day 6; CS at these concentrations appeared to delay rather than inhibit the molting process. Control cultures of *S. equinus* L3 showed no molting at day 6 and 41 ± 9 % molting to L4 by day 10 (Fig. 2C). Molting of these larvae was significantly inhibited at day 10 by cystamine at 1 and 0.5 mM ($P < 0.05$). MDC at the same concentrations appeared lethal to these larvae during their culture from day 6 to 10.

Table I illustrates an average increase in growth measurements of these L3 to L4 cocultured with inhibitors. These values were obtained by comparing measurements at the initiation of culture and at 10 days of culture. The results indicate marked reduction in larval sizes by MDC and CS. Also, an abnormal enlargement of anterior (buccal) region was noticed in *S. edentatus* L3 that did not molt to L4 stage larvae (Fig. 3).

EFFECT OF TGASE INHIBITORS ON L4 VIABILITY AND GROWTH

Cystamine and MDC were added at appropriate concentrations on day 6 to *S. vulgaris*, on day 11 to

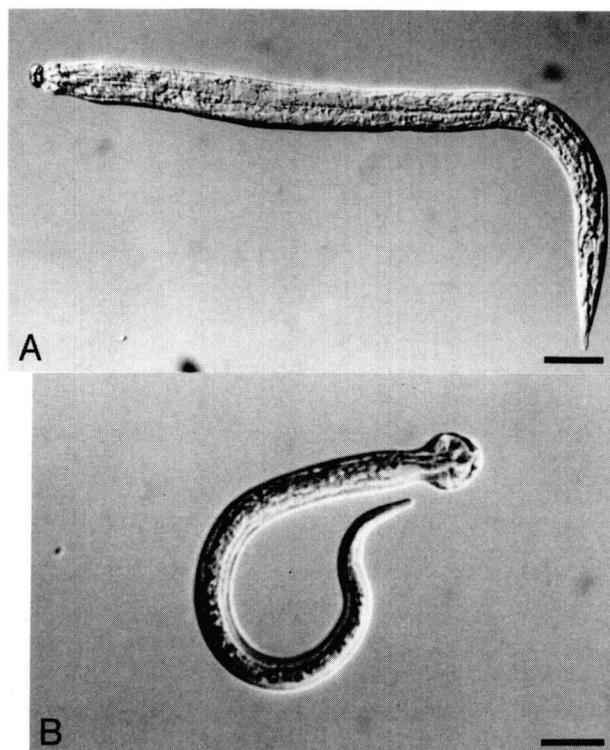


Fig. 3. – Effect of CS on *Strongylus edentatus* (L3). Control L3 cultured under normal conditions as described in methods section (A); L3 cultured with 0.5 mM CS at day 10 showing an abnormal anterior end (B). Scale bar = 10 µm.

S. edentatus, and on day 6 to *S. equinus* L4 cultures. Viability and growth were measured on days 8, 10, and 13 (Fig. 4A) for *S. vulgaris* and *S. equinus*, and on days 12 and 18 (Fig. 4B) for *S. edentatus*. These results indicate that CS had less effect on viability of these parasites at all the concentrations tested. However, MDC at high concentrations showed an inhibitory effect on viability of L4. It is interesting that both the inhibitors significantly reduced the L4 growth as determined by length and width measurements (Table II).

Inhibitor	Conc (mM)	<i>S. edentatus</i>			<i>S. equinus</i>		
		Width ¹	length ²	% inhibition in length ³	Width ¹	Length ²	% inhibition in length ³
Cystamine (CS)	0.5	154.7	0.0	100	97.1	0	100
	1.0	88.7	13.4	56	42.9	0	100
Monodansylcadaverine (MDC)	0.5	66.7	24.9	19	14.3	2.5	26
	1.0	56.7	23.4	24	0	0	100
None	–	193.3	30.6	–	117.1	3.4	–

* Growth was determined by morphometric measurements on the width (¹) and length (²) of larvae from day 6-10.

³ : % inhibition = % reduction of average length of larvae cultured with inhibitor as compared to average larval length in medium alone controls.

Table I. – Increase (%) in growth* of *Strongylus edentatus* and *S. equinus*- L3 to L4 in cultures containing transglutaminase inhibitors.

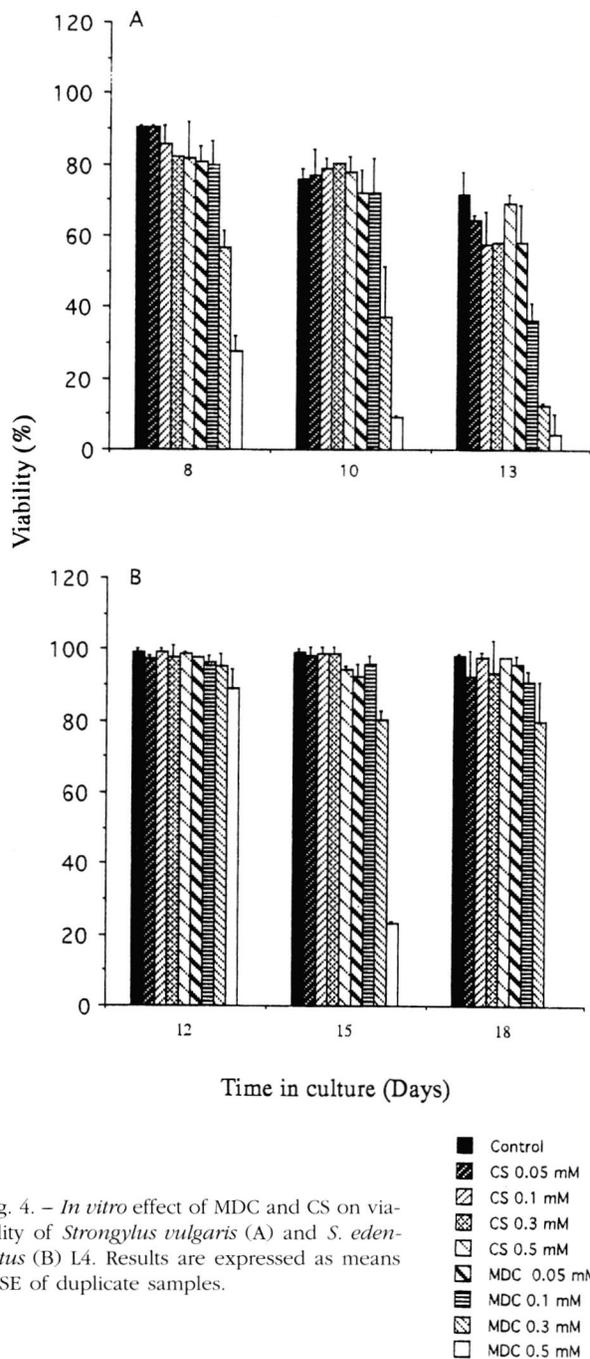


Fig. 4. – *In vitro* effect of MDC and CS on viability of *Strongylus vulgaris* (A) and *S. edentatus* (B) L4. Results are expressed as means \pm SE of duplicate samples.

DISCUSSION

Transglutaminases are a family of enzymes have been shown to be involved in the posttranslational modification of proteins. The presence of a biologically active TGase in nematodes was first reported in filarial worms and its catalytic activity was attributed to the growth and development of microfilarial stages (Mehta *et al.*, 1990, 1992). Significance of TGase catalyzed crosslinking of proteins in viability,

growth and development, and microfilarial production was implicated (Rao *et al.*, 1991). Transglutaminase activity was also shown to play a role during molting of L3 to L4 stages of *O. volvulus* (Lustigman, 1993). Biochemical evidence suggests that the presence of TGase catalyzed cross linking of γ -(glutamyl)isopeptides in the maturation of sheath and cuticle of filarial nematodes (Mehta *et al.*, 1992; Tarcsa *et al.*, 1992; Zahner *et al.*, 1995; Conraths *et al.*, 1997). It is interesting that the mammalian enzyme is quite distinct from the parasite enzyme in substrate specificity, enzyme activity and molecular mass (Mehta *et al.*, 1992; Singh *et al.*, 1995; Lustigman *et al.*, 1995).

In the present study we have shown the ubiquitous nature of this enzyme by identifying its activity in different stages of strongyle parasites and in adult worms of two other gastrointestinal nematodes of equids. The cytosol preparations from crude extracts of *B. malayi* and *D. immitis* and partially purified enzyme preparations of parasite-TGase and their specific inhibition of enzyme activity by using various inhibitors clearly suggest the biological activity of this enzyme in cross linking of parasite proteins (Singh & Mehta, 1994; Lustigman *et al.*, 1995; Singh *et al.*, 1995). However, effect of inhibitors on the enzyme activity of purified protein of strongyles is unknown. Recently, the TGase enzyme from dog filarial nematode was cloned and showed no sequence homology to any known TGases (Chandrashekar *et al.*, 1998). Whether TGase from strongyles also represents a novel protein remains to be determined. Nevertheless, these results suggest that TGase of nematodes could serve as an effective target enzyme to develop effective broad spectrum chemotherapeutic agents against a wide range of nematode parasites.

We used two specific inhibitors to show their inhibition of biological activity of TGase in viability, molting, and growth of L3 to L4 of *Strongylus* species *in vitro*. For this purpose, CS that inactivates the enzyme activity by binding to the active site and MDC, a competitive amine substrate inhibitor that inhibits specifically the endogenous protein crosslinking by TGase (Lorand *et al.*, 1979; Folk, 1980; Castelhana *et al.*, 1990; Auger *et al.*, 1993) were used at millimolar concentration. The results indicate that neither inhibitor affects larval viability of *S. vulgaris* and *S. equinus*. *Strongylus edentatus* viability was affected when incubated at high concentrations of MDC. Similarly, the MDC effect on molting of L3 to L4 is greater when compared with CS and the latter appears to delay the molting process of *S. vulgaris* L3. Moreover, the effect on viability and molting within the species was varied. For example, *S. edentatus* and *S. equinus* were more sensitive to CS and MDC effects than *S. vulgaris*. Both the inhibitors seemed lethal to the *S. equinus* and *S. edentatus* L3

Inhibitor	Conc (mM)	<i>S. edentatus</i>			<i>S. vulgaris</i>			<i>S. equinus</i>		
		Width ¹	Length ²	Inhibition in length (%) ³	Width ¹	Length ²	Inhibition in length (%) ³	Width ¹	Length ²	Inhibition in length (%) ³
Cystamine (CS)	0.05	34	25	42	15.7	29	31	63	24	52
	0.1	27	26	39	15.7	20	52	50	14	72
	0.3	16	35	18	15.7	18	57	44	0	100
	0.05	20	32	25	15.7	25	40	0	0	100
Monodansylcadaverine (MDC)	0.5	20	26	39	15.7	13	69	25	0	100
	0.1	14	34	21	6.2	25	40	0	0	100
	0.3	14	8	81	15.7	7	83	0	0	100
	0.5	D	D	–	D	D	–	0	0	100
None	–	27	43	–	25.3	42	–	50	50	–

* Growth measurements were made morphometrically on the width (¹) and length (²) of larvae.

^a: measurements were made from day 10-19; ^b: from day 6-15; ^c: from day 14-20.

D: Dead.

³: % inhibition = % reduction of average length of larvae cultured with inhibitor as compared to average larval length in medium alone controls.

Table II. – Growth* measurements of *Strongylus vulgaris*^a, *S. edentatus*^b and *S. equinus*^c L4's in cultures containing transglutaminase inhibitors.

during molting phase to L4 when used at higher concentrations. The inhibition of molting indicates that TGase catalyzed crosslinking may be involved in the formation of a new cuticle and any failure in this biochemical process may affect the molting process. These results are in agreement with previous observations of TGase inhibition leads to the inhibition of *O. volvulus* L3 molting (Lustigman *et al.*, 1995). However, CS was less effective in inhibiting *S. vulgaris* larval molting and this could be due to the species variation in type and nature of cuticular makeup and transcuticular absorption. Apparently, strongyle larvae are more fastidious than filarial nematodes as they can withstand high temperatures and enzymes when compared with their comparable stages of filarial nematodes. For example, strongyle larvae are resistant to a wide range of adverse conditions for prolonged periods (Ogbourne & Duncan, 1985). Therefore, the concentration of inhibitors needed to test the viability and molting of *Strongylus* spp. was greater than what has been used for such studies with *Brugia* or *Onchocerca*.

Evidence from ultrastructural studies suggests that in *O. volvulus*, these two inhibitors did not affect the new L4 cuticle formation during L3 growth to L4, but the cuticle was inseparable at some junctions on the surface from the old cuticle during ecdysis (Lustigman *et al.*, 1995). These authors also have noticed the TGase catalyzed cross linked protein, ε-(γ-glutamine)lysine in developing L3 by immunoprecipitation studies strongly suggesting the biological activity of this enzyme in molting process. In this study under normal conditions, shedding of the cuticle began at the anterior end of emerging L4's from the old cuticle and leaving behind

these cuticle casts in culture medium. However, strongyle larvae particularly *S. edentatus* L3's incubated with the active site inhibitor CS failed to molt to L4's showed an abnormal bulging of the anterior end suggesting that at optimal concentrations, TGase inhibitors may inhibit the release of larval cuticle. Therefore, the developing larvae begin to enlarge within the old cuticle, by that causing an abnormal cephalic bulge. In contrast, the other two species failed to show such an abnormal development, which could be explained as species variation in cuticular turnover and initiation signals for ecdysis. Effect on L4 viability with MDC and growth with both the inhibitors strongly supports the notion that TGase is involved in growth and development of these nematodes. N-bezyloxy-carbonyl-D,L-b-(3-bromo-4,5-dihydroisoxazol-5-yl)-alanine benzylamide (Syntex Inc., Palo Alto, CA), another TGase inhibitor that like CS binds to the active site of TGase (Auger *et al.*, 1993; Castelhan *et al.*, 1990), also inhibited *O. volvulus* larval molting (Lustigman *et al.*, 1995) and *Nippostrongylus braziliensis* L4 viability and molting to adult (Castelhan *et al.* 1990). Although these studies have identified the presence of TGase enzyme in a wide range of nematode species, additional studies are needed to determine the regulatory activity of TGase in molting process and worm development in vivo (Geary *et al.*, 1995). Secondly, what triggers the enzyme activity in protein cross linking during the growth of these parasites is unknown.

In summary, these results strongly support the presence of TGase in parasitic nematodes and its involvement in molting and growth of larval stages. TGase thus

represents an important regulatory enzyme that may be targeted for antiparasitic studies by the use of highly potent parasite-specific enzyme inhibitors.

ACKNOWLEDGEMENTS

We are thankful to the support of Onchocerciasis Control Programme in West Africa, United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases, Macrofil Chemotherapy Project (KM).

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Reçu le 7 décembre 1998

Accepté le 27 mars 1999