Trypanosoma evansi Sialidase: Surface Localization, Properties and Hydrolysis of Ghost Red Blood Cells and Brain Cells-Implications in Trypanosomiasis

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A membrane-bound sialidase was isolated from blood stream (BS) *Trypanosoma evansi* partially purified and characterized. The enzyme is a glycosyl phosphatidyl inositol (GPI) membrane anchored protein. It was solubilized from *T.evansi* cells recovered from infected camel blood by detergent treatment with Triton CF 54 and partially purified by a series of chromatography steps. The enzyme was optimally active at pH 5.5 and 37 °C. It had a K_M and V_{max} values of 4.8×10^{-6}M and 3.75×10^{-6} mol/min.mg protein with Neu5Acα2, 3lac as substrate respectively. The K_M and V_{max} values with fetuin (4-nitrophenyl-oxamic acid) as substrate were 2.9×10^{-2}M and 4.2×10^{-3} mol/min.mg protein in the same respect. Kinetic analysis with methly umbelliferyl sialate (MU-Neu5Ac) gave K_M and V_{max} values of 0.17 mM and 0.84 mmol/min.mg protein respectively. The *T. evansi* SD could hydrolyse internally linked sialic acid residues of the ganglioside GM_2 , but was inactive towards colomic acid, and Neu5Ac2, 6. lac. When ghost red blood cell (RBC) was used as substrate, it desialylated the RBC in the following order of efficiency; mouse, rat, camel, goat, and dog. Similarly, cerebral cells isolated from BalbC mouse was desialylated by the *T. evansi* SD.
Inhibition studies using 2-deoxy-2, 3 didehydro-*N*-acetyl neuraminic acid (NeuAc2, 3en) against MU-Neu5Ac revealed a competitive inhibition pattern with K_i of 5.8 μM. The enzyme was also inhibited non-competitively by parahydroxy oxamic acid (pHOA), and competitively by *N*-ethylmaleimide and *N*-bromosuccinate with K_i values of 25, 42, and 53 μM, respectively. It was activated by Mg^{2+} ion and inhibited by Cu^{2+} and Zn^{2+}.

Key words: Trypanosoma evansi, Sialidase, Kinetic Properties

Introduction

Trypanosoma evansi is a haemoflagellate protozoa that infects domestic animals causing animal trypanosomiasis. Anaemia is one of the principal signs of the acute phase of trypanosomiasis in livestock (Muray and Dexter, 1988; Esievo and Saror, 1991). Haematological analysis of experimentally infected coatis (*Nasua nasua*) with *T. evansi* showed an intense anaemia which followed the first wave of parasitaemia (Haerrera *et al.*, 2002). In *T. vivax* infection, the occurrence of sialidase, an enzyme that hydrolyses a wide range of glycoconjugates to release sialic acid, has been implicated in the etiology of anaemia during trypanosomiasis (Esievo, 1983). Removal of the negatively charged sialic acids from the exposed epitopes of surface molecules is consequential to the changes in the behaviour of cells leading to their uptake and clearance by macrophages (Jancik and Schauer, 1974; Durocher *et al.*, 1975).

The strong correlation of the serum sialic acid and parasitaemia during *T. vivax* experimental infection in cattle underscores the importance of sialidase in the pathophysiology of animal trypanosomiasis (Esievo *et al.*, 1982). Both *Trypanosoma evansi* and *Trypanosoma vivax* are the most extensively distributed trypanosomes responsible for diseases in livestock. Moreover, western blot anaylsis and indirect immunofluoresence assays show high immunological cross reaction between these two parasites (Uzcanga *et al.*, 2002).

So far sialidase have been described in bacteria and viruses and their roles in the pathology of infection well documented (Tanaka *et al.*, 1992). In protozoa, sialidases have been reported in *T. congolense*, *T. brucei*, *T. vivax*, *T. rhodesiense*, *T. rangelli* and *T. cruzi* (Esievo, 1983; Reuter *et al.*, 1987; Engstler *et al.*, 1992; Nok and Uemura, 1998). However while the role of the enzyme in *T. cruzi* has been linked to cell invasion, (Schenkman *et al.*, 1991) in the south American Chagas disease, a definitive role in the African trypanosome is yet

to be elucidated. Moreover, most reports have shown the existence of the enzyme in only the procyclic insect forms of the trypanosome parasite and its absence in the blood stream stage which are infective to mammals. Because anaemia in *T. vivax* infection correlates with the rise in serum free sialic acid, the anaemia in *T. evansi* infection could produce the same effect on account of their immunological similarities. In this report, we show for the first time the existence of a novel blood stream *T. evansi* sialidase and describe some of its characteristics which could be related to the pathophysiology of animal trypanosomiasis.

Materials and Methods

Trypanosomes, chemicals, enzyme

Trypanosoma evansi STIB 731-AA (IL 1392) was obtained from an abbatoir owned by the State Government in Kano City of Nigeria, used throughout the experiments.

Fetuin Sepharose, Q-Sepharose and Sephadex G75, and GM_2 were products of Sigma Chemical Co. Fetuin, *N*-(4-nitrophenyl-oxamic acid), Neu5Acα2,3lactose, Neu5Ac2en, PI-PLC (*Bacillus cereus* were purchased from Sigma, St. Louis, U. S. A. Neu5Acα2,6lactose was a generous gift from Prof. Dr. Roland Schauer (Kiel, Germany).

Purification of sialidase

Blood stream *T. evansi* parasites were isolated from a sick camel at a cell density of 2×10^6 cells/ml. The cells were separated using the Lanham and Godfrey method (1970), centrifuged at $10000 \times g$ for 10 min and washed in phosphate buffered saline (PBS), pH 7.2. About 5 vol of 0.2%Triton CF 54 were dispensed into the cells and left at room temperature for 20 min and then centrifuged at $5000 \times g$ for 20 min. After three extractions, no activity was detected in the cells. The combined extracts was passed through a Q-Sepharose column (2×20 cm) and eluted by a gradient of 0.01–0.3 M phosphate buffer, pH 6.9. Sialidase active fractions were pooled and concentrated by pressure dialysis with a 20 kDa-membrane molecular exclusion limit (Sartorius Göttingen, Germany.) The concentrate (5-ml) was chromatographed on Fetuin-Sepharose column (2×10 cm) equilibrated with 66 mM phosphate buffer, pH 6.9 and eluted with 0.1 M α mannoside. The recovered protein was dialysed and used as the partially purified enzyme. Protein was quantified as described by Bradford (1976).

pH and temperature studies

The activity of the enzyme was assayed between 10–50 °C at pH 5. A pH-dependent assay was also performed at 37 °C using 50 mM acetate pH 4–6, 0.1 M phosphate buffer pH 6.2–7.2 and 0.1 M Tris-glycine pH 7.5–9.5 at 40 °C.

Enzyme assays

Sialidase was routinely assayed at 37 °C using 0.2 mM MU-Neu5Ac (Reuter *et al.*, 1987). Substrate specificity studies were conducted with the following substrates: Neu5Acα2,3lactose, Neu5Acα2,6lactose, Neu5,9Ac_2α2,3lactose, fetuin, transferin, colomic acid and mucin as described by Warner and O'Brien (1979). Briefly the substrates were adjusted to 1 mM bound Sialic acid and incubated with 0.5 Unit of the enzyme in 800 ml reaction mixture for 2 h. At the end of the incubation the released sialic acid was quantified (Schauer, 1987).

Kinetic experiments were performed with Neu5Acα2,3lac (0.1–0.7 mM), MU-Neu5Ac at (0.1–0.7 mM) and Fetuin (0.1–1.5 mM). The inhibition experiments were done in the presence of 0.05 mM of the inhibitors; Neu5Ac2en and pHOA using the substrate MU-Neu5Ac. Inhibition experiments were also conducted using 20 μM para-chloromercuribenzoate (pCMB) in the presence and absence of 0.05 mM dithiothreitol was also used. The effects of the chlorides of the following divalent cations on the activity of the enzyme were studied in the presence of (1–5 mM) Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}.

Preparation of ghost red blood cells (RBC) and mouse brain cells. Ghost RBC were prepared from; dog, rat, mouse, camel and human blood as described by Dodge *et al.*, (1963). The isolated cells were suspended in a 0.5 ml phosphate buffered saline (PBS) pH 6.8 and incubated with 0.5 units of the partially purified SD at 37 °C for 3 h. At the end of 1 h, the reaction was terminated and the released Sialic acid analysed as described by Schauer and Corfield (1982). A similar reaction system was set up in the presence of 0.05 mM

Neu5Ac2, 3en (sialidase specific inhibitor). At the end of the reaction, sialic acid was quantified as done previously (Schauer, 1987).

Brain cells were prepared by sacrificing a healthy BalbC mouse. Its brain was dissected aseptically to remove the cerebellum and homogenized in 50 mM acetate buffer, pH 6. Aliquot of 0.5 ml of the brain cell suspension was then incubated with 0.5 units of the *T. evansi* SD for 3 h in the presence of 0.01% Triton X100. At the end of the reaction the released SA was quantified as done previously. A parallel reaction was set up in the presence of 0.05 mM of the SD inhibitor Neu5Ac2,3,en and used as control to check whether hydrolysis is enzyme-linked.

Surface localization

Trypanosoma evansi cells (10^6) were lysed at 0 °C for 1 h in 200 µl of 10 mM Tris/HCl (pH 7.4) containing 140 mM NaCl and 2% Triton X-114. After centrifuging, the hydrophilic and hydrophobic portions were obtained as described by (DiasFilho *et al.*, 1995). Briefly, the supernatant was dispensed into 6% w/v sucrose (1:1.5 w/v) and incubated at 37 °C for 1 h. The hydrophilic and hydrophobic phases were recovered above and below the cushion respectively after centrifugation at $1000 \times g$ for 10 min. Both phases were assayed for sialidase activity. Similarly, osmotically lysed *T. evansi* cells were incubated in the absence and presence of 5 U/ml of PI-PLC from *B. cereus* or 5 mM $ZnCl_2$ for 2 h at 37 °C before phase separation with Triton X-114.

Results and Discussion

Osmotic lysis of the *T. evansi* cells with the nonindet Triton X-114 resulted into the partitioning of an aqueous and detergent phases with a predominance of the SD activity in the aqueous phase. A similar distribution was observed when the parasites were initially treated with phosphatidyl inositol-phospholipase C specific (PI-PLC) prior to the phase separation. The induced release of the sialidase in both cases was significantly inhibited by the addition of 0.5 mM of $ZnCl_2$ (Fig. 1). This suggests that the enzyme is associated with the plasma membrane by interaction involving the GPI-anchor. Since $ZnCl_2$ (PLC inhibitor) inhibited the release of sialidase, it could imply that during the course of trypanosomiasis an endogenous PI-PLC might be involved in the release of the enzyme. This contrasts with the *T. brucei* sialidase which is resistant to PI-PLC as well as being GPI linked (Engstler *et al.*, 1992). The deviation in this property could be a developmental stage specific character, since the *T. brucei* SD was from the procylic insect form of the parasite. Since it is GPI-linked, the release of the *T. evansi* SD during the course of infection may be triggered by the action of host phospholipase C or D along with the endogenous PLC from dead parasites. *Trypanosoma evansi* was collected from infected camel blood at a density of about 10^6/ml and used for the quantitative extraction of sialidase. After solubilization, the enzyme was purified by about 310 fold to apparent homogeneity using a series of chromatography steps as shown in Table I. The enzyme did not bind to the Q-Sepharose column. However it served to separate other contaminants and cell lysates giving a 20-fold purification. The most effective of the purification steps was the Fetuin-sepharose column, which yielded a 310-fold purification with enhanced stability. The interaction between this column with the enzyme could imply that it is glycosylated since the sialyl residues on the fetuin motif may form strong covalent interaction with the exposed possibly galactosyl residues on the *T. evansi* SD.

The pH dependent profile of the *T. evansi* sialidase revealed an optimal peak at pH of 6.5 with a

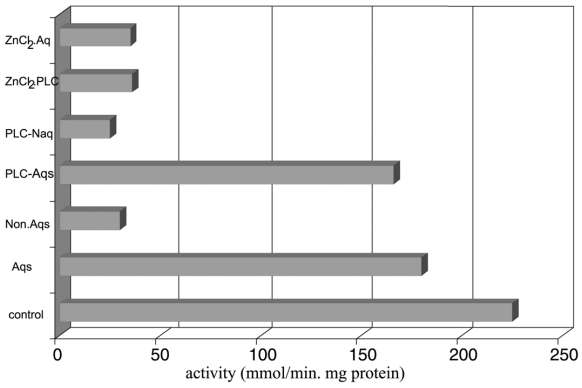

Fig. 1. Surface localization showing the distribution of *T. evansi* SD on a sucrose cushion under different conditions in the presence and absence of phospholipase C (PLC from *Bacillus cereus*) and $ZnCl_2$ as described in the Materials and Methods section. Data from three experiments were used to plot the graph.

Table I. Purification of sialidase from *Trypanosoma evansi*. Results are expressed as averages of three experiments. All steps were conducted at 4 °C.

Purification steps		Specific activity [μmol/min.mg protein]	(Protein) [mg]	Activity [μmol/min]	Purification	Yield %
I	Sedimented cells	5.8	250	1450	1	100
II	Osmotic lysis	17	118	1363	3	94
III	Q-sepharose chromatography	116	25	1073	20	74
VI	Fetuin-sepharose chromatography	1798	4.2	174	310	12

complete cessation of activity at pH 4 and 8.5 (not shown). An acidic pH optimum region will contribute to the enhancement of the enzymatic reaction of the *T. evansi* SD by general acid catalytic mechanism. The control of intracellular concentration of H^+ is a very essential pathological character of *T. evansi.* Indeed the parasite has a very sensitive pH intracellular pool which is released by the ionophore nigericin (Mendoza *et al.*, 2001). Both *T. brucei* and *T. congolense* sialidases have pH optimum at 6.5–7.2 while the American *T. cruzi* sialidase has a pH optimum that falls in the range 6.0–6.5 (Reuter *et al.*, 1987; Engstler *et al.*, 1992; Cross and Tackle, 1993). Bacterial and viral sialidases have been shown to have pH optimum activity at 4–5 (Crennel *et al.*, 1993; Uzcanga *et al.*, 2002). The contrasting pH profile could be organism related.

Results of the temperature dependence studies showed that the *T. evansi* sialidase activity increased with temperature and was optimal at 37 °C. Data from the temperature studies subjected to Arrhenius plot of log initial velocity as a function of reciprocal absolute temperature gave an activation energy of 12 KJ/mol. Such a low activation energy is thermodynamically the favourable implying less frequency of collision is required to surmount the activated complex and form products. This is very important for the enzyme, considering the mucinuous glycoconjugate microenvironment, which will tend to lower, the rate of contact between the enzyme and the glycoconjugate substrate.

The enzyme was very sensitive to organomercurial reagents loosing about 50% of activity in the presence of 65 μM *p*-chloromercuribenzoate. The kinetic inhibition analysis gave a K_i value of 40 μM (Table II). The inhibition by *p*-CMB was completely blocked in the presence of 0.05 mM of dithiothreitol showing the importance of the -SH group in the catalytic activity of the *T. evansi* sialidase. The group specific compounds *N*-bromosuccinamide and *N*-ethylmaleimide competitively inhibited *T. evansi* SD in the micromolar range (Fig. 2a,b). The evaluation of the kinetic data gave K_i of 42 and 53 μM, respectively (Table II) showing the strong nature of the interactions. Since these compounds are specific for histidine and tryptophan, respectively, both amino acids could be important catalytic components in the active site of

Table II. Kinetic Parameters of *Trypanosoma evansi* sialidase in the presence of different substrates and inhibitors. Results were derived from the averages of three experiments. The activity of the enzyme was assayed at 37 °C and pH 6.5.

Substrate/Inhibitors	K_M [μM]	V_{max} [mmol/min.mg protein]	V_{max}/K_M[min^{-1}]	K_i [μM]
Neu5Acα2,3lac	4.9	3.75	14	
Neu5Acα-MU	0.17	0.84	7	
Fetuin	2.9	4.25	0.35	
Neu5Ac2,3en	–	–	–	5.3
pHOA	–	–	–	2500
pCMB	–	–	–	40
N-bromosuccinamide	–	–	–	42
N-ethylmaleimide	–	–	–	53

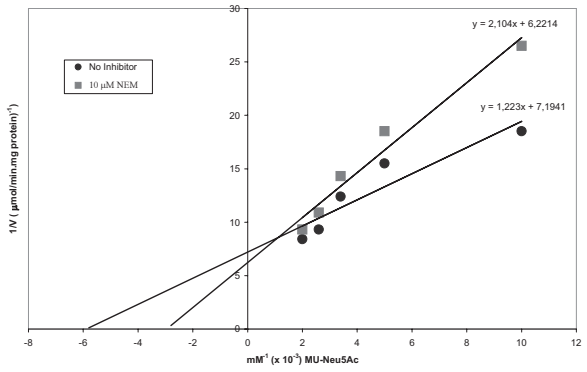

Fig. 2.a. Line Weaver Burk plots of initial velocity data for the determination of inhibition on *Trypanosoma evansi* SD by 10 μM *N*-ethyl maleimide (NEM) using methyl umbelliferyl sialate (MU-Neu5Ac) as substrate. Data from three experiments were used to plot the graph using the MS Excel program.

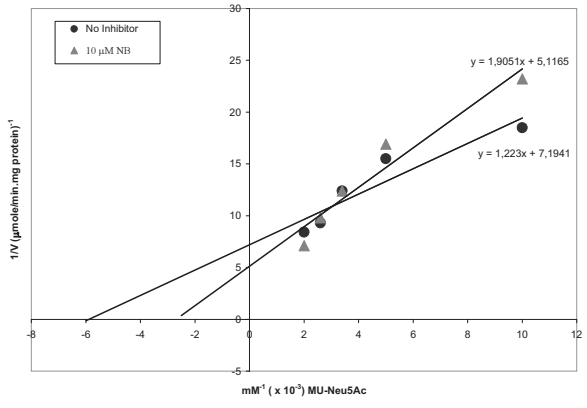

Fig. 2.b. Line Weaver Burk plots of initial velocity data for the determination of inhibition on *Trypanosoma evansi* SD by 10 μM *N*-bromosuccinamide (NB) using methyl umbelliferyl sialate (MU-Neu5Ac) as substrate. Data from three experiments were used to plot the graph using the MS Excel program.

the *T. evansi* enzyme. Further analysis on the specific positions of these amino acids by site directed mutagensis could be beneficial in the design of anti-SD of *T. evansi* for the control of anaemia.

In the analysis of the effect of divalent metal ions on the activity of the enzyme, only Mg^{2+} was found to activate the enzyme by 55% Cu^{2+}, Fe^{2+} and Zn^{2+} were fairly inhibitory to the enzyme activity (not shown) while, Ca^{2+} had no influence on the activity of the enzyme. The presence of these ions in physiological systems, could modulate the activity of the *T. evansi* enzyme during the course of infection. Interestingly, *T. evansi* has been reported to have an effective system of Ca^{2+} control to resolve fast Ca^{2+} signals .Measurement of the basal Ca^{2+} in *T. evansi* indicate that homeostatic mechanism maintains Ca^{2+} at 106 nM in the presence of 2 mM calcium (Mendoza *et al.*, 2001).

Several complex carbohydrates and oligosaccharides were assayed for substrate specificity. As shown in Table III, the relative hydrolysis rate compared to MU-Neu5Ac show that the *T. evansi* sialidase has a preference for α 2,3-linked sialic acids. The compound Neu5Acα2,3 lactose was the best amongst the physiological substrates. The substrate Neu5,9Ac_2α2,3 lactose was hydrolysed at a rate of 40% that of Neu5Acα2,3 lactose. The diminished rate of hydrolytic activity on Neu5,9-Ac2,3lac suggests *O*-acetylation as a possible hindrance to SD-catalysed hydrolysis of cells with a high proportion of *O*-acetylation. This appear to corroborate the observation on the trypanotolerance nature of the Ndama cattle, known to have a higher proportion of *O*-acetylated sialic acids than its the susceptible Zebu counterpart. (Esievo *et al.*, 1990; Shugaba *et al.*, 1994). Several workers (Corfield *et al.*, 1981; Engstler *et al.*, 1993) have reported the phenomenon of resistance towards sialidase hydrolysis due to *O*-acetylation on the parent glycoconjugate. The substrates MU-Neu5Ac, fetuin, mucin and transferin were also readily desialylated by the enzyme. This is a property shared by most sialidases which are specific for α,2,3-linked sialic acids (Corfield *et al.*, 1981; Engstler *et al.*, 1993). The ganglioside (GM_2) was

Table III. Results of substrate specificity of partially purified *T. evansi* sialidase.
The substrates were adjusted as described in the Material and Method section to contain 1 mM of bound sialic acid in 60 mM acetate buffer, pH 6.5. The results are averages of three determinations.

Substrate	**Product (Neu5Ac) [μmol/h·mg protein]**
MU-α-Neu5Ac	365 ± 44
Neu5Ac2,3-α-lactose	318 ± 60
Neu5,9$Ac_2$2,3-α-lactose	42 ± 25
Neu5Ac2,6-α-lactose	125 ± 66
Colomic acid	50 ± 29
Fetuin	218 ± 65
Mucin	194 ± 45
Transferrin	105 ± 56
Ganglioside GM_2	115 ± 36

also hydrolysed by the enzyme albeit at a slower rate. This feature is different to the properties of other protozoal sialidases generally known to hydrolyse only terminally linked α,2,3-linked sialic acids (Esievo *et al.*, 1990; Shugaba *et al.*, 1994; Corfield *et al.*, 1981; Engstler *et al.*, 1993; Pereira, 1983). The ability of the *T. evansi* SD to hydrolyse GM_2 is an advanced biochemical property over other protozoal SD which restrict their actions on just α2, 3 terminal sialyl residues. This observation could imply some critical differences in primary structure between the *T. evansi* SD and those from other protozoa. Furthermore, it suggests it could have the proerty to affect cell types with internally located sialyl residues. The substrates, colomic acid and Neu5Ac2,6lac, were not desialylated by the *T. evansi* proving that α,2,6 and α,2,8-linked sialic acids are unaffected by the enzyme. Sialidases from *T. congolense*, *T. bruceri*, *T. rangelli* have been reported to exhibit similar characteristic (Reuter *et al.*, 1987; Engstler *et al.*, 1993; Pontes deCarvalho *et al.*, 1993). Kinetic analysis of the enzyme performed under steady state conditions gave K_M values of 0.17, 4.9 and 2.9 μM for MU-Neu5Ac, Neu5Acα2,3 lactose and fetuin, respectively (Table 2). The enzyme was strongly inhibited competitively by Neu5Ac2,3en with a K_i of 28 μM. The inhibition by *N*-(4-nitrophenyl) oxamic acid was relatively weak with a K_i of 2.5 mM. Similar findings were reported on *T. brucei* (Engstler *et al.*, 1993, 1995; Pontes de Carvalho *et al.*, 1993). Both inhibitors are specific for most sialidases studied. The competitive inhibition against the *T. evansi* SD shows that the constituents of its active site could be similar in composition to those of bacteria and viral SD.

Ghost erythrocytes prepared from the different animal sources were desialylated by the enzyme (Fig. 3). The mouse RBC was more susceptible to the enzyme suggesting that it contains a relatively higher amount of α-2,3 linked sialic acids than the other RBCs. This in part also explains the fast rate of the development of parasitemia in mice than the other animals during the preliminary phase of our experiments to raise the *T. evansi* parasites. When the incubation was done in the presence of the SD-specific inhibitor (Neu5Ac2,3en) there was no desialylation thus ruling out the possibility of an artefact as source for the hydrolysis. This is a further indication of the potential application of

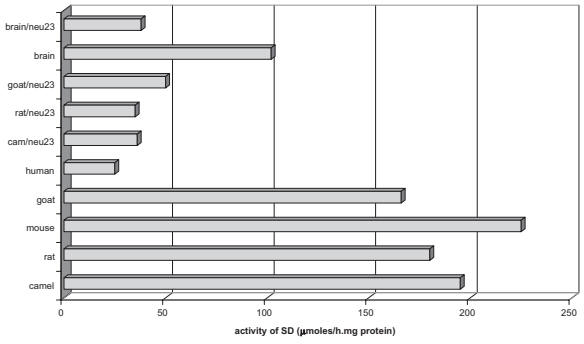

Fig. 3. Substrate specificity studies on *Trypanosoma evansi* SD using ghost red blood cells (RBC) from mouse, rat, goat, camel and human and bran cells from mouse. In each case, the reaction was conducted in the presence or absence of the sialidase inhibitor Neu5Ac2,3en.

SD inhibitors in the control of anaemia. The higher rate of hydrolysis in the dog, mouse and rat than the camel's RBC could also be attributed to the higher proportion of α-2,3-linked sialic acids. The human RBC was the least susceptible to the *T. evansi* sialidase, which could partly explain why humans are no natural host, to *T. evansi.* The desialylation of RBC by the parasite SD is a prelude to anaemia, since circulating macrophages can easily recognise the exposed galactosylated epitopes of asialo-RBC and clear them away from circulation. The ultrastructural study on mice experimentally infected with *T. evansi* was reported to lead to muscle fibre changes including atrophy, autophagic vacuoles and mononuclear infiltration formed by macrophages suggesting that the skeletal muscle is an important target tissue for *T. evansi* (Finol *et al.*, 2001). This characteristic pathological feature could commence by an initial removal of the sialyl residues from the tissues. When the cerebral cells from the BalbC mice were subjected to hydrolysis, they were readily desialylated by the *T. evansi* sialidase. The main source of sialic acids in the brain tissue is the ganglioside and are internally located in the oligosaccharide chain of the glycolipid. Together with the results on substrate specificity studies show that the enzyme can hydrolyse internally linked sialic acid residues. The desialylation of the brain cells was inhibited by Neu5Ac2.3en confirming that the hydrolysis is an enzymatic process. Gangliosides are very vital components of cells and are highly enriched in neurons and errors in their synthesis or catabolism

could lead to disease syndromes generally referred to as ganglio*did*osis (Gravel *et al.*, 2001). The high rate of susceptibility of the brain cells and GM_2 to the *T. evansi* SD could have far reaching consequences in the development of trypanosomiasis if the parasites cross the blood brain barrier. Gangliosidosis could thus be another feasible pathology in chronic trypanosomiasis.

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