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Trans-sialidase and sialidase activities discriminate between morphologically indistinguishable trypanosomatids

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The expression of trans-sialidase and sialidase activities in the kinetoplastid protozoa was explored as a potential marker to discriminate between the morphologically indistinguishable flagellates isolated from human, insects and vertebrate reservoir hosts. By virtue of the differences observed in the ratios of these enzyme activities, a collection of 52 species and strains comprising the major taxa of these parasites could be separated into four expression types. Type-I parasites express comparable levels of both trans-sialidase and sialidase activities (*Endotrypanum* species and *Trypanosoma lewisi*). Type-II parasites express predominantly trans-sialidase activity (*Trypanosoma cruzi* and *Trypanosoma conorhini*).Type-III parasites express sialidase activity exclusively (*Trypanosoma rangeli* and *Trypanosoma leeuwenhoeki*). Type-IV parasites do not express either activity (*Leishmania* species and *Trypanoplasma borreli*). The measurement of trans-sialidase and sialidase activities thus permits the differentiation of parasites frequently found in the same insect vectors that are difficult to distinguish, such as *T. cruzi* and *T. rangeli*, or in the same sylvatic vertebrate and invertebrate hosts, such *as Leishmania* and *Endotrypanum*.

Trans-sialidases are a class of recently described cell-surface-exposed *trans*-glycosidases that differ from the Golgi-associated sialyltransferases in that they catalyze the transfer of *N*-acylneuraminate (sialic acid) residues from substrates other than cytidine monophosphate (CMP)-sialic acid to glycoproteins, glycolipids and polysaccharides. Like all *trans*-glycosidases, the trans-sialidases also exhibit hydrolase (i.e. sialidase/neuraminidase) activity in the absence of suitable acceptors.

Trans-sialidase activity was discovered in the protozoan parasite *Trypanosoma cruzi* (order *Kinetoplastida*, family *Trypanosomatidae*) [1–5], the agent of Chagas' disease in the American continent, and shortly after in their African counterparts *Trypanosoma brucei ssp.* [6, 7], the causative organisms of sleeping sickness in humans and of nagana in domestic mammals. Trans-sialidases are, however, not restricted to trypanosomes as they also occur in the non-pathogenic trypanosomatids of the genus *Endotrypanum* [8], and in the pathogenic fungus *Pneumocystis carinii* (L. Trimble, N. Pavia & M. E. A. Pereira, unpublished results). Although unequivocal evidence for the specific biological roles of the trans-sialidases in these parasite systems is lacking, the *T. cruzi* trans-sialidase is believed to be involved in the process of invasion of mammalian cells [3, 4], in defense against complement attack [9], in the intracellular fate and survival of the parasite (reviewed in [10–14]).

Currently, it is difficult to discriminate between pathogenic and non-pathogenic trypanosomatids. Striking similarities at the morphological, molecular and biological levels exist between many trypanosomatids isolated from sylvatic insects and/or vertebrate reservoir hosts that make the identification of the medically important parasites demanding (reviewed in [15, 16]). Moreover, the geographical distributions of the different vectors and vertebrate reservoirs overlap. For example, the neotropical tree sloths (Edentata; genera *Choleopus* and *Bradypus*), which are reservoirs of at least six *Leishmania* species pathogenic for humans (reviewed in [17]), are hosts of *Endotrypanum* (*E. monterogeii* and *E. schaudinni*; reviewed in [18]) and two *Trypanosoma* species (*T. rangeli* [19] and *T. leeuwenhoeki* [20]), none of which are pathogenic for humans. These parasites species have also been isolated from recently fed anthropophilic sandflies (*Lutzomyia spp.*) [19–24].

Endotrypanum parasites are unique among the Kinetoplastida in that they infect erythrocytes of the vertebrate host [18], but these forms are rare in naturally or experimentally infected sloths. Diagnosis usually relies on the examination of parasites from cultures or from sandflies, and those forms of the parasite are morphologically indistinguishable from the promastigotes of *Leishmania* [25, 26]. Similarly, *T. cruzi* and *T. rangeli* can be found in the same vertebrate and insect hosts [19, 27, 28]. Although the blood-stage forms of *T. rangeli* differ morphologically, they are exceedingly rare in

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Abbreviations. MeUmb-NeuAc, 2′(4-methylumbelliferyl)–D-*N*-acetylneuraminic acid.

Enzymes. Sialidase (EC 3.2.1.18); trans-sialidase.

the circulation in the large majority of the infected populations. In contrast, the parasites obtained from the gut of the vectors and from culture are not easily distinguishable solely on the basis of morphology.

Even though several methods have been proposed to discriminate these trypanosomatids [29–40], the general concensus is that they are cumbersome and not satisfactory, and that the definitive identification can be only attained by recombinant DNA procedures [41–45]. Here, we show that the measurement of sialidase and trans-sialidase activities also permits rapid diagnosis of culture forms of trypanosomatids of medical importance.

MATERIALS AND METHODS

Parasites

Identification of the isolates, their geographic origin and the source of the stocks used in this study, are given in Table 1. Unless otherwise indicated, parasites were grown at 25 °C in Schneider's *Drosophila* medium [46] supplemented with 20% heat-inactivated fetal bovine serum. *T. cruzi* and *T. rangeli* were cultured as described previously [47]. *Trypanosoma lewisi* was grown in Sprague-Dawley weaning rats that received a 0.5-ml inoculum from a frozen sample (provided by J. Bienen and A. Clarkson, Jr, NYU Medical Center, USA) by intraperitoneal injection. Bloodstream forms were isolated from freshly drawn blood (4 days after infection) by centrifugation through a Ficoll:Paque (Pharmacia) cushion layer, as recommended by the manufacturer for the *in vitro* isolation of lymphocytes. Parasites (50%) were recovered from the interphase (lymphocyte layer) or the Ficoll-Paque phase (45%). For the isolation of the contaminant *T. cruzi* strain in the *T. rangeli* San Agustin strain, four two-month-old Swiss-background nude mice (nu/nu, Taconic) were inoculated intraperitoneally with 5×10^7 parasites obtained from *in vitro* cultures. Blood samples were collected every two days and screened fresh for the presence of parasites. Blood smears were stained with Giemsa. Infection of LLC-MK_2 cells was carried out as described by Schenkman et al. [47].

Sample preparation

Cells (1.5 ml 1×10^7–1×10^8 cells ml^{-1}, from 5–8-day-old cultures) were harvested in Eppendorf tubes by centrifugation at $1360\times g$ for 15 min at 4 °C and washed once with ice-cold phosphate-buffered saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4) before lysis. The corresponding culture supernatant was transferred to a fresh tube and cleared by centrifugation at $13000\times g$ for 30 min at 4 °C. The pelleted cells were suspended in lysis buffer (100 mM sodium acetate, pH 7.0, containing 1.5% Nonidet P-40, 0.1 mM *N*-tosyl-L-lysine chloromethane, 0.1 mM phenylmethanesulfonyl fluoride and leupeptin at 25 μg ml^{-1}), at 1×10^8 cells ml^{-1}, by vigorous vortexing followed by a 60-min incubation on ice. Lysates were cleared by centrifugation at $13000\times g$ for 30 min at 4 °C. Protein content was determined by the fluorescamine method, as described by Udenfriend et al. [48], using excitation at 350 nm and reading emission at 460 nm in a Titertek FluorosKan II (Flow Laboratories Inc., McLean, VA, USA). Peptides and polypeptides are known to yield maximum fluorescence near pH 7.0, whereas for other reactive primary amines (amino acids, polyamines, etc.) fluorescence is generally maximal at pH 9.0 with little fluorescence at pH 7.0.

Enzymic activities

Sialidase activity was assayed by the hydrolysis of the fluorogenic substrate 2′(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MeUmb-NeuAc, Sigma) followed by quantification of the fluorescence emitted by methyl-umbelliferone, with excitation at 350 nm and reading emission at 460 nm in the Titertek FluorosKan II. The final reaction mixtures were 10 μl lysate (regularly containing 10 μg protein) and 40 μl 1× substrate cocktail [0.2 mM MeUmb-NeuAc, 0.005% ultrapure bovine serum albumin (Boehringer Mannheim Corporation), in 100 mM sodium acetate, pH 7.0] or 40 μl supernatant and 10 μl 5× substrate cocktail. Maximal-activity values were achieved after an overnight incubation in the dark, at room temperature. The overnight incubation did not increase the blank, background or noise values, which never exceeded a row value of 70 arbitrary fluorescence units. The blank row values were subtracted from the sample row values before normalization and calculation of the enzymic activity. The reaction conditions were determined experimentally to provide maximal activity values (i.e. highest sample *versus* blank ratios.) We have observed that the ratios of sialidase/trans-sialidase activities are maintained independently of the time length (45 min versus 16 h) and/or temperature (22 °C versus 4 °C) of incubation. The reactions were set up such that the measurements were maintained linear with a dynamic range of 1–1000 pmol standard methyl-umbelliferone and, of 0.5–100 μg protein lysate added. To ensure working under the linear range, we tested only 10 μg protein. As monitored with standard solutions of methyl-umbellyferone, one fluorescence unit corresponded to one pmol methyl-umbellyferone. One unit sialidase activity is the amount of enzyme catalyzing the hydrolysis of 1 μmol MeUmb-NeuAc/min (1 U = 1 μmol · min^{-1}).

Trans-sialidase activity was determined by the transfer of sialyl residues from 3′-sialyllactose (donor/substrate) to [D-glucose-1-^{14}C]lactose (acceptor/substrate), in a 50-μl reaction mixture containing 100 mM sodium acetate, pH 7.0, 50 nmol sialyllactose and 370 pmol (54 Ci/mol) radioactive lactose. The mixture was incubated at room temperature for 45 min. The reaction was then terminated by the addition of 1 ml water and loaded onto a 1-ml QAE-Sephadex A50 column (Pharmacia LKB) equilibrated in water. The radioactive product (sialyl-[D-glucose-1-^{14}C]lactose) was eluted with 1 ml 1 M ammonium formate, and quantified by liquid scintillation counting. One unit trans-sialidase activity is the amount of enzyme catalyzing the transfer of 1 μmol sialic acid from sialyllactose to radiolabelled-lactose/min.

DNA isolation and PCR amplification

Genomic DNA was isolated using the TELT mini-prep method [49]. Diagnostic PCR amplification of the *T. cruzi* species-specific, repetitive, non-transcribed sequence SRE-1, was carried out as reported by Novak et al. [45].

Statistical analysis

The differences in enzyme activity ratios between the stocks were tested for statistical significance ($P<0.01$) by the Student's *t*-test (two-tailed) assuming equal variances.

RESULTS AND DISCUSSION

We measured and compared the levels of sialidase and trans-sialidase activities from both cell extracts and culture

Table 1. Origin and identification of the kinetoplastid reference strains, stocks and isolates employed in this study. Designations. M = Mammalia; BRA = *Bradypus*; CHO = *Choloepus*; HOM = *Homo sapiens*; CEB = *Cebus apella*; I = Insecta: SHA = *Lutzomyia shanonni* s.l. Data were acquired from the culture collections of [1] J. J. Shaw, Instituto Evandro Chagas/Wellcome Parasitology Unit, Belém, PA, Brazil, [2] M. P. Deane and [3] A. M. R. Franco and G. Grimaldi Jr., Instituto Oswaldo Cruz/FIOCRUZ, Rio de Janeiro, RJ, Brazil, [4] American Type Culture Collection, Rockville, MD. USA, [5] A. H. C. S. Lopes, Instituto de Microbiologia/UFRJ, Rio de Janeiro, RJ, Brazil, [6] T. V. Barrett, Instituto Nacional de Pesquisa da Amazonia, Manaus, AM, Brazil, [7] E. S. Garcia, Instituto Oswaldo Cruz/FIOCRUZ, Rio de Janeiro, RJ, Brazil, [8] P. Michels, ILP-TROP, Belgium, [9] A. Clarkson, Jr., New York University, New York, USA. Stock identification of the Brazilian isolates was established by isoenzyme, monoclonal antibodies and molecular karyotype analyses (Franco, A. M. R., Momen, H. and Grimaldi, Jr. G., unpublished results).

Stock	Designation	Species	Locale	Source
Reference strains				
T01	MBRA/CO/00/BV388[a]	*Trypanosoma leeuwenhoeki*	Colombia	1
T02	MCHO/CO/00/CH250[c]	*T. leeuwenhoeki*	Colombia	1
T05	MHOM/BR/00/F strain	*T. cruzi*	Brazil	2
T06	Providencia	*T. conorhini*	Brazil	2
T07	MPD	*T. conorhini*	Brazil	2
T08	M238	*T. sp.* (bat)	Brazil	2
T09	ILP-TROP	*Trypanoplasma borreli*	–	8
T10	30085-Y	*T. lewisi*	U.S.A.	9
T21	SC-1	*T. rangeli*	Brazil	7
T22	SC-58	*T. rangeli*	Brazil	2
T23	SC-72	*T. rangeli*	Brazil	2
T24	SC-61	*T. rangeli*	Brazil	2
T25	San Agustin	*T. rangeli*	Brazil	2
T26	T25 subclone	*T. cruzi*	U.S.A.	–
H01	IMEG/US/72/H.meg	*Herpetomonas megaseliae*	U.S.A.	2
L02	MHOM/BR/75/M4147	*Leishmania guyanensis*	Brazil	3
L03	MCEB/BR/84/M8408	*L. shawi*	Brazil	3
E09	MBRA/PA/00/415P01[b]	*Endotrypanum* sp.	Panama	1
E11	MCHO/CR/62/A-9[c]	*E. monterogeii*	Costa Rica	1
E12	MCHO/BR/88/M11602[d]	*E. schaudinni*	Brazil	1
E49	MCHO/CO/68/473	*Endotrypanum* sp.	Colombia	4
Endotrypanum stocks				
E08	ISHA/BR/80/IM217	*E. schaudinni*	Brazil	2
E10	MBRA/PA/81/1222P82[b]	*Endotrypanum* sp.	Panama	1
E13	MCHO/PA/79/GML-30[c]	*E. schaudinni*	Panama	1
E14	MCHO/BR/80/M6159[d]	*E. schaudinni*	Brazil	1
E15	MCHO/BR/79/M5725[d]	*E. schaudinni*	Brazil	1
E42	MCHO/CR/00/LV88[c]	*E. monterogeii*	Costa Rica	5
E43	MCHO/PA/00/LV59[c]	*E. schaudinni*	Panama	5
E44	MCHO/CO/00/Col[d]	*E. schaudinni*	Colombia	5
E45	MCHO/PA/72/3130	*Endotrypanum* sp.	Panama	5
E46	MCHO/CO/68/473	*Endotrypanum* sp.	Colombia	5
E47	MCHO/BR/80/M6159[d]	*E. schaudinni*	Brazil	5
E48	MCHO/BR/00/LV86[d]	*E. schaudinni*	Brazil	5
Brazilian isolates				
E01	MCHO/BR/89/RO9627[e]	*Endotrypanum* sp.	Rondonia	3
E02	MCHO/BR/89/RO1635[e]	*Endotrypanum* sp.	Rondonia	3
E03	MCHO/BR/89/RO1634[e]	*Endotrypanum* sp.	Rondonia	3
E04	MCHO/BR/89/RO1140[e]	*Endotrypanum* sp.	Rondonia	3
E05	MCHO/BR/89/RO1602[e]	*Endotrypanum* sp.	Rondonia	3
E06	MCHO/BR/89/RO1471[e]	*Endotrypanum* sp.	Rondonia	3
E07	MCHO/BR/89/RO1583[e]	*Endotrypanum* sp.	Rondonia	3
E16	MCHO/BR/85/IM2260[d]	*Endotrypanum* sp.	Pará	6
E17	MCHO/BR/85/IM2382[e]	*Endotrypanum* sp.	Rondonia	6
E18	MCHO/BR/85/IM2384[e]	*Endotrypanum* sp.	Rondonia	6
E19	MCHO/BR/85/IM2389[e]	*Endotrypanum* sp.	Rondonia	6
E20	MCHO/BR/85/IM2404[d]	*Endotrypanum* sp.	Amazonas	6
E21	MCHO/BR/89/IM3605[e]	*Endotrypanum* sp.	Rondonia	6
E22	MCHO/BR/89/IM3606[e]	*Endotrypanum* sp.	Rondonia	6
E29	ISHA/BR/80/IM1111	*Endotrypanum* sp.	Amazonas	6
E31	MCHO/BR/85/IM2259[d]	*Endotrypanum* sp.	Pará	6
E32	MCHO/BR/85/IM2380[e]	*Endotrypanum* sp.	Rondonia	6
E33	MCHO/BR/85/IM2393[e]	*Endotrypanum* sp.	Rondonia	6
E36	MCHO/BR/89/IM3603[e]	*Endotrypanum* sp.	Rondonia	6

[a] Host was *B. tridactylus*; [b] host was *B. infuscatus*; [c] host was *C. hoffmanni*; [d] host was *C. didactylus*; [e] host was *C. juruanus*.

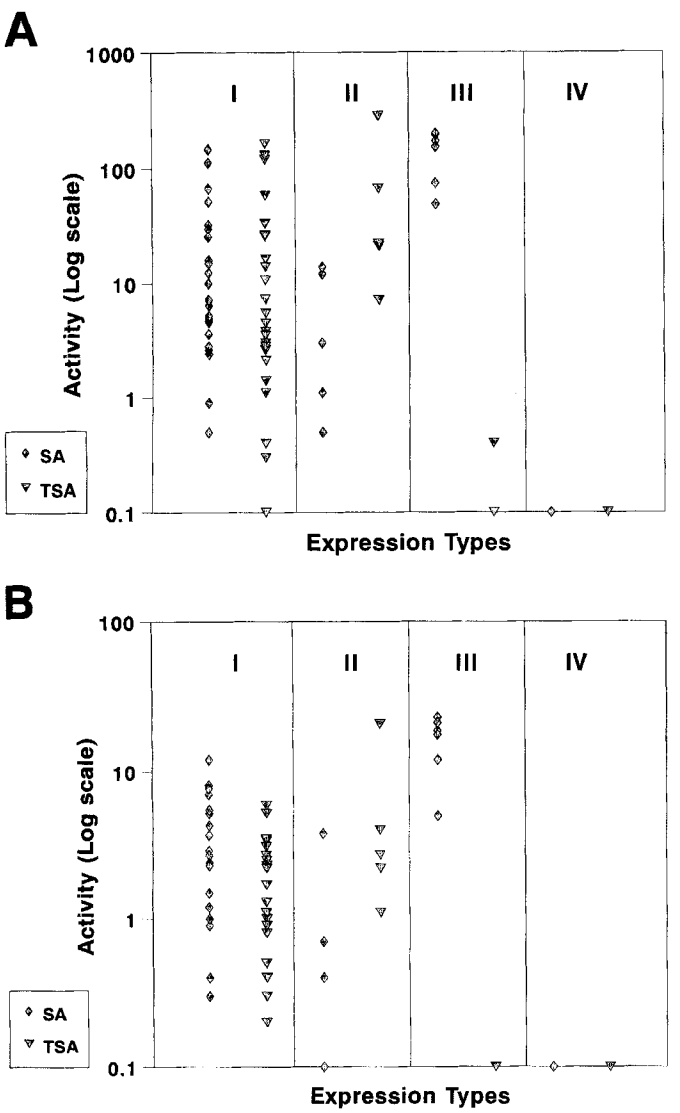

Fig. 1. Levels of sialidase (SA) and trans-sialidase (TSA) enzyme activities in cell lysates (A) or culture supernatants (B) of the kinetoplastids listed in Table 1. Shown is a distribution along the *y*-axis of the activity levels in the four (I–IV) parasite expression types. The *y*-axis is drawn in logarithmic scale. The relative enzymic activity in each sample is expressed as μU mg protein^{-1}. Each value represents the mean number of duplicates from one of three independent experiments. Variations between duplicates and experiments were less than 5%.

Table 2. Sialidase and trans-sialidase expression types in culture forms of trypanosomatids. The activity ratios shown are from cell lysates. Within each expression type, the differences between the ratios from lysates and supernatants are not statistically significant ($t = P > 0.1$).

Type	Activity ratios (sialidase/trans-sialidase)	Parasite
I A[a]	0.97 ± 0.15	*Endotrypanum schaudinni* *Endotrypanum* spp. *Trypanosoma lewisi*[b]
B	2.27 ± 0.86	*E. monterogeii* *E. schaudinni* *Endotrypanum* spp.
II	0.10 ± 0.06	*Trypanosoma cruzi* *T. conorhini* *Trypanosoma* sp. (bat)
III	74.7/0.0 49.2/0.1 180.3 ± 18.0/0.0	*T. leeuwenhoeki* *T. leeuwenhoeki* *T. rangeli*
IV	0.0/0.0 as in [8]	*Trypanoplasma borreli* *Leishmania guyanensis* *L. shawi* *Herpetomonas megaseliae* *L. mexicana, L. donovani, L. major, L. chagasi, L. amazonensis, H. samuelpessoae, Phytomonas davidi, Leptomonas seymouri, Crithidia fasciculata, C. deanei, Blastocrithidia culicis*

[a] The differences between the ratios in A and B are statistically significant ($t = P < 0.01$).

[b] Blood stages of the parasite (see Materials and Methods).

supernatants of 52 parasite stocks comprising the two suborders of the kinetoplastida, *Trypanosomatina* (family *Trypanosomatidae*; genera *Leishmania*, *Endotrypanum*, *Trypanosoma* and *Herpetomonas*) and *Bodonina* (family *Cryptobiidae*; genus *Trypanoplasma*), Table 1. The stocks, which were originally isolated from different hosts (human, wild and domestic animals or sandflies) and geographic areas, were selected in order to represent the pathogenic and non-pathogenic parasites that are morphologically indistinguishable, i.e. within the genus *Trypanosoma*, we examined both pathogenic (*T. cruzi*) and non-pathogenic (*T. lewisi* [50], *T. conorhini* [51], *T. rangeli* and *T. leeuwenhoeki*) parasites.

Fig. 1 depicts the levels of enzymic activities present in cell extracts (A) and culture supernatants (B). The stocks fall into four expression types (I–IV), according to the sialidase/trans-sialidase enzyme activity ratios shown in Table 2. Type I is represented by 23 *Endotrypanum* stocks, which express comparable levels of both activities. Two subgroups can be distinguished: (A) in which the activity ratios are 0.97 ± 0.15 (13 stocks), and (B) in which the ratios are 2.27 ± 0.86 (11 stocks), Table 2. Note, in addition, that the absolute levels of enzymic activities within each subgroup vary greatly (from 0.1–164 μU · mg^{-1}), Fig. 1. The reason(s) for these variations is unknown. Artifacts such as differences in the growth stage of the parasites or proteolytic degradation of the enzyme during sample preparation are unlikely; all *Endotrypanum* extracts were prepared from 5-day-old cultures, and the ratios of the activities were remarkably constant, regardless of the incubation conditions (time length and temperature) of the assays. The marked differences in the levels of sialidase and trans-sialidase enzyme activities observed among the *Endotrypanum* stocks may be genetically determined and perhaps represent strain-specific traits of a species complex [18, 52]. The heterogeneity of *Endotrypanum* stocks was also noted by Franco et al. [36] who analyzed 27 of the 35 *Endotrypanum* stocks used here by isoenzyme electrophoresis. A comparison of the electrophoretic profiles with those from reference strains representing the genus revealed that the enzymic loci were highly polymorphic.

Type II includes *T. cruzi* and *T. conorhini*, which predominantly express trans-sialidase activity. The ratio of siali-

dase/trans-sialidase activities in extracts or culture supernatants of these parasites is 0.1 ± 0.06.

Type III, represented by *T. rangeli* and *T. leeuwenhoeki* stocks, is characterized by high levels of sialidase activity and no, or very little, trans-sialidase activity. In stock T02, we consistently obtained 0.1 $\mu U\ mg^{-1}$ trans-sialidase, a value which falls close to the sensitivity limit of the assay.

Type-IV parasites do not express either activity. Included in this category are the *Leishmania* species, *Herpetomonas megaseliae*, *Trypanoplasma borreli* and 11 stocks that were originally identified as *Endotrypanum*. These 11 stocks differ from the *Endotrypanum* reference strains by morphological, karyotypic, isoenzymic and *in vitro* growth rate criteria ([36], and Franco et al., unpublished observations). Moreover, two of these stocks (E46 and E47) came from reference strains that expressed both activities (E49 and E14, respectively). These observations argue against the authenticity of the non-expressing stocks as belonging to the genus *Endotrypanum*, and suggest that the original stocks were contaminated or mislabelled.

In short, sialidase and trans-sialidase activities were detected in all reference *Endotrypanum* strains (Type I), independently of the reservoir host or the geographic area of isolation, while promastigotes of all *Leishmania* species examined ([8], and this report), including *L. guyanensis* (which shares with *Endotrypanum spp.* both the sloth and sandfly hosts), lack both activities (Type IV).

We have also extended the previous observations that the *T. rangeli* sialidase(s) lacks trans-sialidase activity [40, 53], while the *T. cruzi* enzyme exhibits both sialidase and trans-sialidase activities [5]. We assayed extracts of epimastigotes from five different strains of *T. rangeli* and found that they do not express detectable trans-sialidase activity.

The discriminatory power of the enzymic assays used here is best illustrated by the analysis of *T. rangeli* San Agustin strain (stock T25, Table 1). We found that stock T26 of this strain contained high levels of trans-sialidase activity and, suspecting contamination with *T. cruzi,* we performed several additional tests. Injection into nude mice yielded high levels of parasitemia, and the bloodstream trypomastigotes were morphologically indistinguishable from *T. cruzi*. This *T. rangeli* stock infected LLC-MK cells *in vitro*, and multiplied intracellularly as amastigotes. An axenic culture of the exiting trypomastigotes at 28 °C did not contain the pleomorphic parasite population typical of *T. rangeli* stocks T21–T24, but was morphologically indistinguishable from *T. cruzi*. The bloodstream forms, the tissue-culture trypomastigotes and the epimastigote thus generated from strain T26, all expressed trans-sialidase activity. To confirm independently that these forms were *T. cruzi*, we carried out a differential PCR analysis. *T. cruzi* DNA was detected by amplification of the *T. cruzi*-specific ribosomal spacer repetitive element SRE-1 [45] which is absent in the genome of *T. rangeli* (not shown).

Our results are in good agreement with those reported by Schottelius [39] and Reuter et al. [40], who also distinguished *T. rangeli* from *T. cruzi* epimastigotes using a sialidase fluorescence test on culture supernatants. However, it should be noted that, unless cloning of the parasites is carried out, the fluorescence test cannot discriminate *T. cruzi* from *T. rangeli* in mixed infections or contaminated cultures, (i.e. high levels of sialidase activity reflect either the presence of *T. rangeli* exclusively or a mixed infection with *T. cruzi*). The measurement of trans-sialidase activity, as illustrated above, resolves this diagnostic problem.

An obvious point raised here that awaits further experimentation is the biological significance of the trans-sialidase activity in the culture forms of *T. conorhini* and *T. leeuwenhoeki* for which neither an intracellular lifestyle, nor a pathogenic activity in the mammalian hosts have been described. Similarly, both sialidase and trans-sialidase activities (1 : 1 ratio) are expressed in the blood stages of *T. lewisi*, a rodent parasite that does not enter hosts cells.

Another important issue to be resolved is the sialylation states of the membrane glycoconjugates of Type-I parasites. It is conceivable that there is a dynamic equilibrium between the sialylated and desialylated states. In contrast, some of the surface components may not be accessible to the sialidase activity (if this is a separate enzyme) and they remain mostly sialylated in the presence of suitable donors.

Lastly, the lack of sialidase/trans-sialidase enzyme activities in the epimastigote forms of the cryptobiid *T. borreli* poses a challenging problem on the origin(s) of the trypanosomal enzymes. Current phylogenetic trees inferred from comparisons of nuclear small-subunit rRNA sequences from several kinetoplastids [54–58] indicate that *T. borreli* branches off early from the trypanosomatid lineage. Hence, the occurrence of the sialidase and trans-sialidase genetic information in the trypanosomes may have taken place after the separation of these lineages. Alternatively, the ancestor of *T. borreli* may have lost the information during evolution. Further comparative studies will be needed to address the antiquity of these enzymes and, in particular, whether or not they are present in the bodonids and the deeper rooted neighbours, the euglenoid flagellates.

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Supplementary Material. ***Trans*-sialidase and sialidase activities discriminate between morphologically indistinguishable trypanosomatids.** The raw data used to generate Fig. 1 are available, upon request, from the Editorial Office. A total of two pages are available.