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ORIGINAL PAPER

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Circulating trans-sialidase activity and trans-sialidase-inhibiting antibodies in *Trypanosoma cruzi*-infected mice

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Abstract Parasite-derived trans-sialidase (TS) activity was demonstrated in the serum and blood of *Trypanosoma cruzi*-infected mice. Serum TS activity levels correlated well with parasitemia in BALB/c and Swiss mice during the initial stages of the infection. However, in later stages the TS activity levels decreased despite increasing parasitemia. This coincided with the appearance of circulating TS antibodies. On the other hand, there was always a good correlation between TS activity and parasitemia in athymic nude mice. Sera from mice with high parasitemia and low TS activity inhibited TS activity in vitro. The inhibition was also observed with purified serum IgG, and it was absorbed by staphylococcal protein A, indicating that it was caused by anti-TS IgG antibodies. These antibodies inhibited the enzymatic activity of insolubilized TS, indicating that they act by interfering with the catalytic site rather than by aggregating the enzyme. The presence of inhibitory antibodies, however, did not prevent the progression of parasitemia in BALB/c mice.

Introduction

Some trypanosomatids, including *Trypanosoma cruzi*, express unusual sialyltransferases that catalyse the transfer of (α2-3)-linked sialic acid to terminal β-galactopyranosyl residues on glycoconjugates (Schenkman et al. 1991; Vandekerckhove et al. 1992; Chaves et al. 1993; Pontes de Carvalho et al. 1993; Medina-Acosta et al. 1994). These enzymes, named trans-sialidases (Schenkman et al. 1991), may play important roles in parasite-host interactions. Thus, the trans-sialidase (TS) of the cell-derived, bloodstream *T. cruzi* trypomastigote catalyzes the formation on the parasite surface of a sialyllated epitope, Ssp-3 (Schenkman et al. 1991), which has been shown to mediate attachment and penetration of the parasite into host cells (Schenkman et al. 1992a). There is also evidence that TS is involved in the process by which *T. cruzi* trypomastigotes escape from the host cell phagolysosome into the cytoplasm (Hall et al. 1992) and that it may function as a surface receptor for sialyllated ligands of the host cell membrane (Ming et al. 1993).

TS has an amino-terminal, catalytic domain and a carboxy-terminal domain containing amino acid repeats (Pereira et al. 1991; Uemura et al. 1992; Parodi et al. 1992). The latter domain is considered to be immunodominant, since most TS antibodies are believed to be directed against the repeats (Cazzulo and Frasch 1992). It has been proposed that this immunodominance would be advantageous to the parasite in that it would prevent the formation of antibodies against the catalytic site (Cazzulo and Frasch 1992).

The enzyme is anchored to the parasite plasma membrane by a glycosylphosphatidylinositol tail (Schenkman et al. 1992b) and is shed in culture supernatants in vitro (Libby et al. 1986; Schenkman et al. 1992b) and in the host cell cytoplasm (Frevert et al. 1992). Moreover, it is found as a circulating antigen, namely, the soluble acute-phase antigen (SAPA), in patients with acute Chagas' disease (Affranchino et al. 1989; Parodi et al. 1992).

In this study we investigated the kinetics of appearance of TS activity in the circulation and the formation of TS-blocking antibodies in *T. cruzi*-infected mice as well as their possible relationship with the course of the infection.

Materials and methods

Parasites

Colombian- (Rossi et al. 1984) and Y-strain (Silva and Nussenzweig 1953) *Trypanosoma cruzi* trypomastigotes were derived from LLC-MK2 cell (CCL-7; American Type Cell Cultures Collection, Rockville, Md.) cultures (Schenkman et al. 1991). Y-strain

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T. cruzi trypomastigotes were also maintained by passage of parasites in BALB/c mice; infected blood was kept in liquid nitrogen in the intervals between experiments.

Experimental infection

Female 2-month-old BALB/c, Swiss, or athymic nude mice (nu/nu, BALB/c background) from Jackson Laboratories (Bar Harbors, Me.) were used as indicated below. Groups of 4 mice from each strain were inoculated with 2.5×10^3 Colombian- or Y-strain *T. cruzi* trypomastigotes. Blood samples were collected every other day starting on day 5 postinfection. Levels of parasitemia were quantified by counting the parasites present in fresh blood samples in a hemocytometer.

Assay for TS activity

Either 10 µl of serum or 10 µl of total blood samples previously solubilized with 1% NP40 was incubated with a TS substrate mixture consisting of 50 nmol of α(2-3)-sialyllactose (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 0.36 nmol of [D-glucose-1-^{14}C]-lactose (60 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), and 0.2% bovine serum albumin (BSA, 99% pure; Sigma Chemical Co., St. Louis, Mo.) diluted in 40 µl of 50 m*M* N-2 hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES, pH 7.0) for 12 h at 4 °C. The transfer of sialic acid residues to the radiolabeled lactose was quantified by anion-exchange chromatography on QAE-Sephadex (Sigma Chemical Co., St. Louis, Mo.) minicolumns as described previously (Schenkman et al. 1991). TS activity was expressed in microunits; 1 TS µU corresponds to the amount of enzyme necessary to sialyllate 1 pmol of radiolabeled lactose per minute in an assay containing 360 pmol of radiolabeled lactose and 50 nmol of sialyllactose in a total volume of 50 µl of 20 m*M* HEPES (pH 7.0) at room temperature (Medina-Acosta et al. 1994).

Assay for TS-inhibitory activity

For this assay, TS was insolubilized onto nitrocellulose paper disks of 2-mm diameter to prevent aggregation of the enzyme by possible TS-binding inhibitors. The disks were coated with TS by overnight incubation at 4 °C with 0.15 *M* phosphate-buffered saline (PBS, pH 7.2) containing 333 µU of TS/ml and were subjected to four washings with PBS. The TS used in this coating procedure was semipurified by concanavalin A-affinity chromatography from the supernatant of *T. cruzi* cultures (Schenkman et al. 1992b).

Next, 15-µl vol. of neat mouse sera or of purified IgG solution (see below) were incubated for 60 min at room temperature with the TS-coated disks to allow the reaction of putative TS inhibitors. The remaining TS activity in these disks was subsequently quantified by incubating the disks with the substrate mixture (0.36 nmol of radiolabeled lactose and 50 nmol of sialyllactose) for 30 min at room temperature in a final reaction volume of 15 µl of 20 m*M* HEPES (pH 7.0). Then, 200 µl of water was added to the reaction mixture and the liquid phase was immediatelly transferred to QAE-Sephadex minicolumns and subjected to anion-exchange chromatography to reveal the transfer of sialic acid to radiolabeled lactose as explained above. In control disks the mouse sera were replaced by PBS, by a 1:4 dilution in 20 m*M* HEPES of a mouse ascites-containing anti-TS monoclonal antibody (mAb) 39 (Schenkman et al. 1992b), or by neat rabbit anti-TS sera. These were raised by immunizing rabbits with purified TS or with a synthetic peptide corresponding to the first 19 amino-terminal amino acids of *T. cruzi* TS (Pontes de Carvalho et al. 1993). When diluted 200 times, these antisera produced distinct bands against TS in Western blots, performed as described below. Results were expressed as TS microunits inhibited per milliliter of serum or per 12 mg of purified IgG (which is approximately the amount of IgG present in 1 ml of mouse serum; Hudson and Hay 1980).

Detection of TS by enzyme-linked immunosorbent assay

This was done by a sandwich enzyme-linked immunosorbent assay (ELISA) technique (Voller et al. 1976). Briefly, wells of polystyrene microtiter plates were coated with protein A-purified mAb 39. The coated wells were then incubated successively with (a) mouse sera diluted 1:10, (b) an anti-TS repeat rabbit serum (Pontes de Carvalho et al. 1993), (c) an anti-rabbit IgG-alkaline phosphatase conjugate, and (d) the fluorogenic substrate 4-methylumbeliferyl phosphate (Sigma Chemical Co., St. Louis, Mo.). The fluorescence was measured in a Titertek Fluoroskan II apparatus (Flow Laboratories Inc., McLean, Va.).

Depletion and purification of IgG from mouse serum

First, 30-µl vols. of mouse sera were mixed with 5 µl of 1 *M* TRIS/HCl (pH 8.8) and passed through 100-µl protein A-Sepharose 4B (Fast-Flow, Sigma Chemical Co., St. Louis, Mo.) microcolumns preequilibrated with 20 m*M* TRIS/HCl/200 m*M* NaCl (pH 8.8). After a washing with the same buffer, the antibodies bound to the columns were eluted with 0.1 *M* sodium acetate buffer (pH 2.9). The eluates had their pH immediately adjusted to 7 with the addition of TRIS/HCl buffer (pH 8.8) and were concentrated in Amicon filters of 30-kDa cutoff (W.R. Grace & Co., Danvers, Mass.). Column flow-throughs and eluates were assayed for inhibition of TS activity as described above. Samples from the same sera, passed through Sepharose 4B microcolumns, were used as controls.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and Western blotting

T. cruzi trypomastigotes were washed once with Hanks' balanced salt solution, boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer, clarified by centrifugation at 14,000 *g* for 5 min, loaded onto 7.5% SDS-PAGE gels, and subjected to electrophoresis under standard conditions (Laemmli 1970). The proteins in the gels were blotted onto nitrocellulose paper, which was subsequently blocked by incubation with 0.15 *M* TRIS-buffered saline (TBS, pH 8.0) containing 1% BSA (TBS-BSA). The blots were then probed with mouse sera collected on various days after infection and diluted 1:100 in TBS-BSA or with ascites containing anti-TS mAb 39 diluted 1:800 in the same buffer. Antibodies bound to antigen bands were finally revealed with the use of an alkaline phosphatase – anti-mouse immunoglobulin conjugate and 5-bromo-4-chloro-3-indolyl phosphate as the substrate (BCIP; Sigma Chemical Co., St. Louis, Mo.).

Results

Parasitemia and TS levels in mouse serum and blood

TS was detected in the sera of *Trypanosoma cruzi*-infected mice starting on day 7 postinfection both in an activity assay (Fig. 1A–H) and in an ELISA (not shown). There was a good correlation (r=0.89) between the levels of TS activity and the levels of TS antigen detected by ELISA. BALB/c, Swiss, and nude mice infected with *T. cruzi* trypomastigotes differed markedly in the way their serum TS activities varied with time. Whereas in nude mice infected with Y-strain *T. cruzi* there was a nearly perfect correlation between TS activity levels and parasitemia up to the death of the animals (Fig. 1E–H), in BALB/c and Swiss mice the enzyme levels decreased

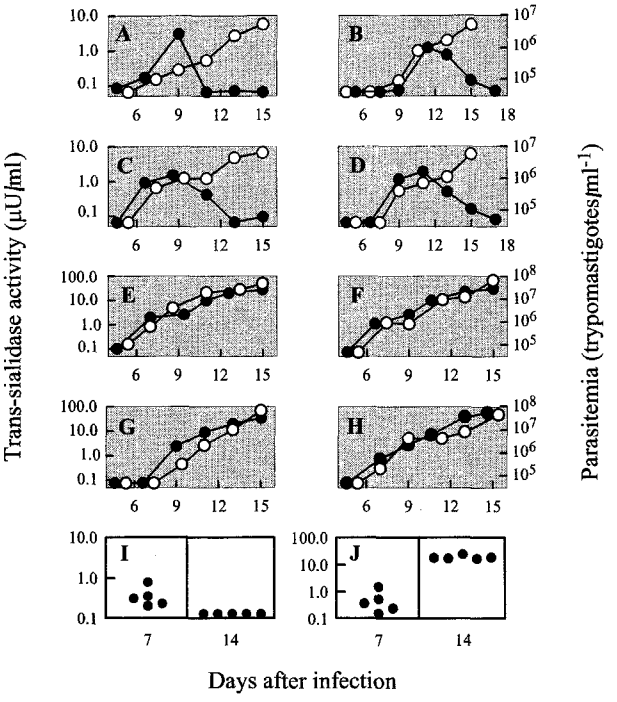

Fig. 1A–J Parasitemia (*open circles*) and TS activity (*closed circles*) detected in the sera and blood of infected mice. Levels of parasitemia were determined in BALB/c (**A, B**), Swiss (**C, D**), and nude (**E–H**) mice. Levels of TS activity were determined in sera from the same mice and in the blood of BALB/c (**I**) and nude (**J**) mice. Each set of curves in **A–H** and each symbol in **I** and **J** represents data obtained from individual mice. Mice were inoculated intraperitoneally on day 0 with 2.5×10^3 Y-strain *Trypanosoma cruzi* trypomastigotes and were bled on the days indicated on the x-axis (**A–H**) or at the *bottom of the panels* (**I, J**). TS activity and parasitemia levels were determined as described in Materials and methods

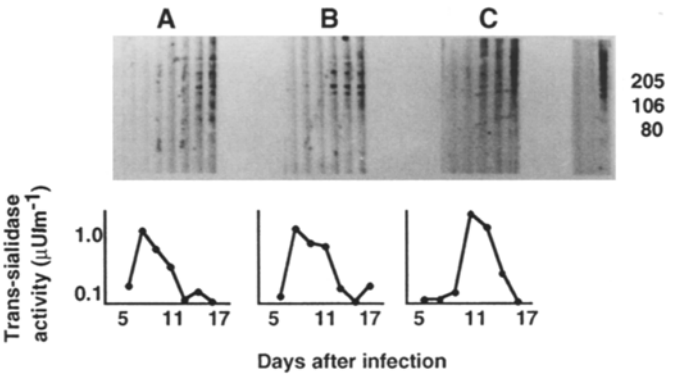

Fig. 2A–C Inverse relationship between TS activity levels and the presence of TS antibodies in the sera of *T. cruzi*-infected mice. TS activity was measured as described in Materials and methods in the sera of Swiss (**A, B**) and BALB/c (**C**) mice infected with 2.5×10^3 *T. cruzi* trypomastigotes on day 0 and bled on the days indicated on the x-axis. The *insets above each graph* show the Western-blotting results obtained with the corresponding sera, diluted 1:100, using as antigen a trypomastigote lysate. The *inset on the right* shows the results obtained with two normal mouse sera, diluted 1:100 (*first two lanes*), or with the monoclonal TS antibody 39, diluted 1:800 (*third lane*). The *numbers on the right* refer to the molecular weight of standard markers

abruptly some days after infection in spite of progressively increasing parasitemia, as shown for some representative mice in Fig. 1A–D. Moreover, TS activity could be detected in solubilized whole blood of infected BALB/c mice on day 7 postinfection but was virtually absent on day 14 (Fig. 1I), despite the observation that the numbers of parasites in the blood samples were sufficient to allow the easy detection of TS in the radioassay employed in this work.

Nude mice infected with Y-strain *T. cruzi* had parasitemia and enzyme levels 10–15 times higher than those of BALB/c mice (Fig. 1). However, mice of both strains died around day 18 postinfection, during the peak of parasitemia, despite the complete absence of TS activity in BALB/c mice. Colombian-strain *T. cruzi* caused in BALB/c mice a parasitemia that eventually reached levels similar to that caused by Y-strain *T. cruzi* between days 11 and 17 postinfection. However, the TS activity in the sera of these mice was always close to background levels (not shown). In Swiss mice the Colombian strain did not produce parasitemia detectable by the method employed in this work, but TS activity could be detected in their sera between days 9 and 11 postinfection (not shown).

Appearance of TS antibodies in mouse serum coincides with reduction in levels of TS activity

As shown for some representative sera in Fig. 2, antibodies recognizing TS were detected by the Western-blot technique in *T. cruzi*-infected BALB/c and Swiss mice from day 13 postinfection onward, i.e., the period when TS activity decreased. As expected, no TS antibodies could be detected in sera from infected nude mice (not shown).

Suppression of TS activity by sera of *T. cruzi*-infected mice

Sera from several infected or noninfected control mice were assayed for their ability to suppress TS activity. The infected sera had suppressive activity significantly higher ($P<0.05$, Wilcoxon's rank-sum test) than that of the normal mouse sera (Table 1). In general, this activity was stronger in later stages of infection. An anti-TS mAb and rabbit antisera against *T. cruzi* TS did not inhibit TS activity (Table 1).

Demonstration that IgG from infected-mouse sera inhibits TS activity

Depletion of IgG from the sera of infected mice reduced their inhibitory activity on TS. Conversely, the IgG purified from the sera inhibited the TS to an extent comparable with those recorded for the nondepleted sera (Fig. 3). The differences in TS-inhibitory activity observed between sera passing through protein A-Sepharose 4B columns and those passing through Sepharose 4B columns

Table 1 Suppression of TS activity by the sera of *Trypanosoma cruzi*-infected mice

Serum donor/mAb	Mouse strain	μU of TS inhibited per ml of serum/ascites[a]
Normal mice[b]	BALB/c	0.7
	BALB/c	2.8
	BALB/c	0.0
	BALB/c	0.0
	Swiss	7.3
MAb 39[c]	–	0.0
Rabbit 16[d]	–	0.0
Rabbit 15[e]	–	0.0
Y-strain *T. cruzi*-infected mice[f]	BALB/c	20.0
	BALB/c	79.6
	BALB/c	57.0
	BALB/c	75.5
	Swiss	22.1
	Swiss	32.5
	Swiss	27.5
Colombian-strain *T. cruzi*-infected mice[g]	Swiss	29.0
	Swiss	29.3

[a] 15 μl of sera or 15 μl of a 1:4 ascites dilution was preincubated for 60 min with TS-sensitized nitrocellulose disks, which were subsequently assayed for TS activity as described in Materials and methods

[b] Mice were inoculated intraperitoneally with saline at 13–18 days or 25 days before serum collection, respectively

[c] Ascites containing the anti-TS mAb 39 (Schenkman et al. 1991)

[d] Rabbit immunized with TS purified from *T. cruzi* culture supernatants (Pontes de Carvalho et al. 1993)

[e] Rabbit immunized with a synthetic peptide corresponding to the first 19 NH2-terminal amino acid residues of the *T. cruzi* TS (Pontes de Carvalho et al. 1993)

[f] Mice were inoculated intraperitoneally with 2.5×10^3 *T. cruzi* trypomastigotes at 13–18 days before serum collection

[g] Swiss mice were inoculated intraperitoneally with 2.5×10^3 *T. cruzi* trypomastigotes at 25 days before serum collection

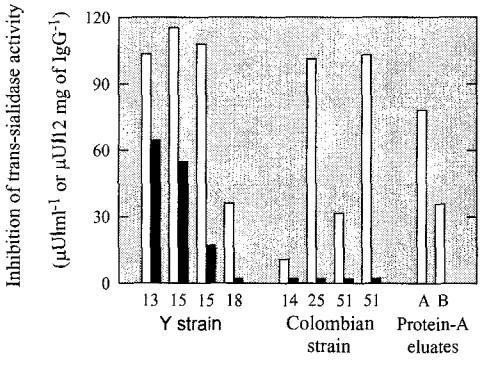

Fig. 3 Inhibition of TS activity by sera of mice infected with *T. cruzi* or by IgG (protein-A eluate) purified from these sera. Sera were tested after absorption with Sepharose 4B (*open columns*) or protein A-Sepharose 4B (*solid columns*). Each set of open and solid columns corresponds to data obtained from individual sera. *Numbers below the columns* refer to the days after infection on which sera were collected. Animals were BALB/c or Swiss mice infected with 2.5×10^3 Y- or Colombian-strain *T. cruzi* trypomastigotes as indicated *below the columns*. Protein-A eluate *A* was obtained from protein A-Sepharose preincubated with the sera producing the results represented by the *first four sets of columns*; protein-A eluate *B* was obtained from protein A-Sepharose preincubated with the remaining sera

were statistically significant ($P<0.05$; Wilcoxon's rank-sum test).

Discussion

The detection by an ELISA of TS in the sera of BALB/c and Swiss mice infected with *Trypanosoma cruzi* is documented in this paper. This confirms previous reports on the presence of SAPA in infected animals (Affranchino et al. 1989; Parodi et al. 1992). However, we also report the detection of TS activity in these sera, which is in accordance with a report of sialidase activity in the serum of a single human patient accidentally infected with *T. cruzi* (de Titto and Araujo 1988). These findings indicate that TS may act on host structures in a systemic way, independently of their contact with the parasite. This could account for the in vivo desialylation of erythrocytes described in *T. cruzi*-infected animals (Pereira 1983).

The progressive reduction in TS activity observed in the sera of *T. cruzi*-infected mice despite increasing parasitemia could be explained by an accumulation of TS-inhibitory factors in their blood. In fact, sera from these mice were capable of inhibiting TS activity in vitro. This inhibitory activity was potent enough to achieve complete inhibition of TS from the circulating parasites themselves, since no TS activity could be detected in whole-blood lysates containing parasites, which had been collected after the 2nd week of infection. That the inhibitory factors were TS antibodies was indicated by the following findings: (1) TS antibodies could be detected in all sera collected after, but not before, the observed decrease in TS levels; (2) TS activity never fell in sera from infected nude mice, which do not produce thymus-dependent antibodies; (3) sera depleted of IgG with insolubilized protein A had markedly reduced inhibitory activities; and (4) IgG purified from these sera inhibited the enzymatic activity of TS.

Inhibition by antibodies could be due to aggregation of the enzyme and/or to interference with its catalytic site, in both cases leading to reduction of its functional concentration. In our assay, the existence of antibodies capable of interfering with the catalytic site of the enzyme was demonstrated, since TS was purposefully insolubilized such that it would not be affected by aggregation. That aggregation was indeed not involved was supported by the finding that not every type of TS antibody inhibited TS activity. Thus, dilutions of ascites containing TS mAb and of rabbit anti-TS sera did not inhibit TS activity, although they contained considerably more TS antibodies than the inhibitory mouse sera, as estimated by titration and/or intensities of bands in Western blots (Fig. 2 and data not shown). Therefore, these monoclonal and polyclonal antibodies very likely recognize TS epitopes unrelated to the enzyme catalytic site. This is consistent with the proposal that the carboxy-terminal, noncatalytic domain of TS is immunodominant (Cazzulo and Frasch 1992). However, as shown in the present paper, it seems that there is no absolute immunodominance in infected mice.

In this study, levels of TS and TS-inhibitory activities were expressed as microunits per milliliter and as microunits inhibited per milliliter, respectively. This facilitated direct comparisons between activities measured in individual mice, allowing estimations of the relative intensity of the inhibition. Thus, the levels of inhibitory activity detected after day 13 of infection in BALB/c mice were 7–28 times higher than the maximal levels of TS activities observed before day 13. This very intense inhibitory activity led to the virtual absence of TS activity in the sera and blood of some infected BALB/c mice (Fig. 1A and 1I) but did not prevent the advance of parasitemia and the disease outcome. In the course of this rather effective anti-TS humoral immune response, it is unlikely that the enzyme would mediate parasite-host interactions in the blood such as the possible migration of *T. cruzi* to tissues through its binding to sialylated ligands on endothelial cells. Further studies are necessary, however, to find out whether modulation by antibody of the TS concentration on *T. cruzi* would influence its time of permanence in the bloodstream. It could be advantageous for parasitism, for instance, that in later stages of the infection TS antibodies would enable parasites to stay longer in the blood such that they would remain available for the blood-sucking insect vector of the disease rather than reinfecting other tissues and killing the host.

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Note added in proof When this paper was being prepared for publication, similar results in patients with Chagas' disease (Pereira-Chioccola et al. 1994) and in *T. cruzi*-infected mice (Leguizamon et al. 1994; Pereira-Chioccola et al. 1994) were reported.

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