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The *trans*-Sialidase from *Trypanosoma cruzi* Induces Thrombocytopenia during Acute Chagas' Disease by Reducing the Platelet Sialic Acid Contents

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Strong thrombocytopenia is observed during acute infection with *Trypanosoma cruzi*, the parasitic protozoan agent of American trypanosomiasis or Chagas' disease. The parasite sheds *trans*-sialidase, an enzyme able to mobilize the sialyl residues on cell surfaces, which is distributed in blood and is a virulence factor. Since the sialic acid content on the platelet surface is crucial for determining the half-life of platelets in blood, we examined the possible involvement of the parasite-derived enzyme in thrombocytopenia induction. We found that a single intravenous injection of *trans*-sialidase into naïve mice reduced the platelet count by 50%, a transient effect that lasted as long as the enzyme remained in the blood. CD43$^{-/-}$ mice were affected to a similar extent. When green fluorescent protein-expressing platelets were treated in vitro with *trans*-sialidase, their sialic acid content was reduced together with their life span, as determined after transfusion into naïve animals. No apparent deleterious effect on the bone marrow was observed. A central role for Kupffer cells in the clearance of *trans*-sialidase-altered platelets was revealed after phagocyte depletion by administration of clodronate-containing liposomes and splenectomy. Consistent with this, parasite strains known to exhibit more *trans*-sialidase activity induced heavier thrombocytopenia. Finally, the passive transfer of a *trans*-sialidase-neutralizing monoclonal antibody to infected animals prevented the clearance of transfused platelets. Results reported here strongly support the hypothesis that the *trans*-sialidase is the virulence factor that, after depleting the sialic acid content of platelets, induces the accelerated clearance of the platelets that leads to the thrombocytopenia observed during acute Chagas' disease.

Sialic acids are abundant nonreducing terminal sugars that decorate mammalian glycolipids and glycoproteins. Because they confer a negative charge that may dramatically change the biologic properties of a given surface molecule, these sugar residues have been implicated in many biological processes, including cell-cell interactions, T-cell activation, and cellular fate. Plasmatic glycoproteins and blood cell oligosaccharides are highly sialylated, and the presence of the sugar is considered a key determinant for their survival in blood. In the case of blood glycoproteins, the loss of sialyl residues tagged them for destruction by liver cells via capture through the asialoglycoprotein receptors. It is known that even a small decrease in the sialic acid contents of platelet and red cell surfaces results in a reduction in the life span (35), and in vitro treatment of platelets with neuraminidase induces accelerated clearance from the blood (23). In addition, injection of neuraminidases into mammals also reduces the half-life of the platelets (10, 19). More importantly, cell surface sialylation is physiologically decreased in several human diseases, resulting in faster removal of platelets from the blood (1, 11, 17, 28). These findings clearly indicate that sialic acid residues are involved in the platelet half-life in blood.

Chagas' disease is an endemic parasitosis that affects about 20 million people, and around 40 million people are at risk of infection in the Americas (40). *Trypanosoma cruzi*, the causative agent, is a flagellated parasite with a complex life cycle involving a hematophagous insect vector and a mammalian host. During the acute phase of *T. cruzi* infection, several hematologically abnormal parameters, including marked thrombocytopenia, are observed (9). These alterations are transient and can be prevented by trypanocidal drugs (9, 27), but there is still no suitable molecular explanation for this. *T. cruzi* is unable to synthesize sialic acids de novo (reviewed in reference 16) but circumvents this limitation by expressing the enzyme *trans*-sialidase (TS), which is able to directly transfer α(2,3)-linked sialyl residues among glycoproteins or glycolipids (16, 34). TS is anchored to the membrane by glycosylphosphatidylinositol (2, 3) and is shed into the surrounding environment, and it is detected in the blood of infected animals and human patients during the acute stage of the infection (14, 24). In the C terminus, the enzyme has tandem repetitive amino acid units that allow it to persist in blood for at least 3 days (5, 6), which allows it to induce pathological disorders even far from the infectious foci or to act on the blood cells (14, 30). One of the biological activities is induction of apoptosis in components of the immune system (25), including CD4$^+$

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$CD8^+$ thymocyte depletion inside the nurse cell complex (29). Accordingly, virulent *T. cruzi* strains shed more TS activity (33). Animals that survive infection and chronic Chagas' disease patients elicit TS-neutralizing antibodies that are able to inhibit TS enzymatic activity and limit the period in which the distributed TS can systemically operate to the early steps of the infectious process. Both the systemic activity restricted to the acute phase and the ability of TS to alter the cell surface sialyl residue distribution are characteristics that are expected for a virulence factor that might be associated with the thrombocytopenia observed during this stage of the infection.

Here we report that the enzyme was able to remove sialic acids from the platelet surface, leading to faster clearance of the platelets from circulation. Furthermore, neutralization of the circulating enzyme by passive transfer of a monoclonal antibody to *T. cruzi*-infected animals prevented the clearance of transferred tracer platelets. Our results support the hypothesis that TS from *T. cruzi* is a virulence factor involved in the thrombocytopenia observed early during the infection, which provides a molecular explanation for the pathological findings.

MATERIALS AND METHODS

Mice. The BALB/cJ, C3H/HeJ, and C57BL/6J mouse strains and green fluorescent protein (GFP)-expressing C57BL/6-Tg(ACTbEGFP)1Osb/J hemyzygous transgenic male mice were originally obtained from The Jackson Labs (Bar Harbor, Maine) and were bred in our facilities. B6.CD43^{null} female mice backcrossed eight times to C57BL/6 mice were generously provided by Anne I. Sperling (University of Chicago) and crossed with GFP-expressing males. F1 $CD43^{+/-}$ GFP-expressing males were backcrossed with $CD43^{-/-}$ females, and green males in the offspring were tested by PCR for the presence of the CD43 gene. A $CD43^{-/-}$ GFP-expressing male was identified and crossed with $CD43^{-/-}$ females to produce $CD43^{-/-}$ GFP-expressing mice. Animals that were 60 to 90 days old were used in this study except where indicated otherwise.

TS. Enzymatically active and inactive TS recombinant proteins that differed by a single $Tyr_{342}His$ point mutation (12) were expressed in *Escherichia coli* DH5α. Proteins were induced with isopropyl β-D-thiogalactopyranoside (Sigma Chemical Co, St. Louis Mo.) and purified to homogeneity by immobilized metal affinity chromatography through Ni^{2+}-charged Hi-Trap chelating columns (Amersham Biosciences, Uppsala, Sweden), followed by ion-exchange chromatography with a MonoQ column (Amersham Biosciences) as described previously (7). The His tag was not removed from the recombinant enzyme. The specific activities of recombinant and natural TS were around 3 to 4 U/nmol of TS protein (1 U of TS activity was defined as the amount of enzyme that transferred 10 nmol of sialyl residue to lactose in 1 min under standard conditions). TS was injected intravenously (i.v.) at the doses indicated below for each experiment in 0.2 ml of phosphate-buffered saline (PBS).

TS assay. The enzymatic activity of purified TS was assayed by measuring the amount of sialic acid residue transferred from sialyllactose (Sigma) to [^{14}C]lactose (Amersham Biosciences) as previously described (24).

***T. cruzi* infection.** Q501/3, a clone of the Tulahuen *T. cruzi* strain (lethal, lineage II), and K-98, a clone of the CA-I *T. cruzi* strain (nonlethal, lineage I) (33), were maintained by serial passage in mice. Animals were inoculated with 250 (Q501/3) or 50,000 (K-98) sanguineous trypomastigote forms by the intraperitoneal route.

Platelet purification. Peripheral blood was collected from the retroocular plexus in polypropylene tubes containing 3.8% (wt/vol) sodium citrate (citrate/blood ratio, 1:9). Platelet suspensions were obtained as described previously (21), with slight modifications. Blood diluted 1:1 in PBS was overlaid onto a density gradient (HISTOPAQUE-1083; Sigma). Platelets were recovered together with the mononuclear cells, diluted 1:1 with PBS, and centrifuged at 140 × *g* for 10 min. The supernatant was centrifuged for 10 min at 1,500 × *g*, and the pellet was resuspended in citrate-treated plasma previously centrifuged at 1,500 × *g* (platelet-poor plasma) or PBS. All the manipulations were carried out at room temperature. The number of platelets was determined by manual counting with a Neubauer hemocytometer.

Sialic acid determination. Platelets (5×10^7 cells) were incubated with TS (0.1 to 6 μg) for 1 h at room temperature in platelet-poor plasma. To determine the α(2,3)-linked sialic acid remaining in the surface, platelets were washed with PBS and incubated with an excess (6 mU/μl) of the specific neuraminidase from *Salmonella enterica* serovar Typhimurium LT2 (New England Biolabs) in 150 mM NaCl—20 mM Tris-HCl (pH 6.8) for 1 h at 37°C. The platelets were centrifuged at 1,500 × *g* for 10 min, and the sialic acid content of the supernatant was determined by the high-performance liquid chromatography–thiobarbituric acid assay (31).

Irradiation of mice. Mice received a lethal dose (9 Gy) of gamma radiation, and 72 h later the animals were inoculated i.v. with 0.5 or 5 μg of TS. Platelet counts were determined 18 h later.

Determination of in vivo survival of TS-treated platelets. GFP-expressing platelets (5×10^7 cells) from a transgenic mouse were incubated with 0.1 to 2 μg of TS for 1 h at room temperature in platelet-poor plasma. The platelet suspension was diluted in 0.2 ml of PBS and injected i.v. Eighteen hours after transfusion, green platelets were counted by using an epifluorescence microscope (Eclipse 600; Nikon, Tokyo, Japan).

Professional phagocyte depletion. Kupffer cells and other macrophages were depleted by injection of liposome-encapsulated clodronate (dichloromethylene bisphosphonate) (4, 41, 42). Animals received 250 μl of a liposome suspension intraperitoneally 24 h before administration of 10 μg of TS. The clodronate was a gift from Roche Diagnostics GmbH, Mannheim, Germany. Phosphatidylcholine (LIPOID E PC) was obtained from Lipoid GmbH, Ludwigshafen, Germany, and cholesterol was obtained from Sigma.

Monoclonal antibody administration. A TS-neutralizing mouse monoclonal antibody (immunoglobulin G2a) was recently obtained in our lab (T. Pitcovsky R. Muiá, and O. Campetella, unpublished data), and ascitic fluid (neutralizing titer, 1:80,000, as determined by a TS inhibition assay [26]) was obtained from animals inoculated with hybridoma. In three separate experiments, mice received a single 50-μl dose of ascitic fluid intraperitoneally on day 10, 11 or 12 after infection, when the parasitemia values were 1×10^5 to 8.5×10^5 parasites/ml, and 24 h later 1×10^8 to 2×10^8 fluorescent platelets were transfused. The remaining fluorescent platelet count was determined 18 h later. Sera from passively transferred animals were able to neutralize 100% of added TS under standard conditions (26).

Splenectomy. Animals (30 to 45 days old) were splenectomized under chloral hydrate (Sigma) anesthesia and left to recover for at least 2 weeks before further analysis.

Statistical analysis. Student's *t* test was employed.

RESULTS

TS is able to reduce the life span of platelets. Several hematological alterations, including strong thrombocytopenia, occur during the acute phase of Chagas' disease (9, 27), the period in which the TS is detected in the circulation. To determine if TS is involved in the induction of thrombocytopenia, different amounts of enzyme (1 to 10 μg) were injected intravenously (Fig. 1A). A strong reduction (around 50%) in the normal platelet count was observed 18 h after TS injection (Fig. 1A). This effect was found to be transient and dose dependent, and a significant reduction was observed even when 1 μg of enzyme was administered. No effect was observed when enzymatically inactive TS was given.

TS has the ability to disseminate systemically through the bloodstream, and the blood and bone marrow are the two main sites where the enzyme may act to reduce the platelet count. From previous assays it seemed that TS was able to affect the platelets in blood. No obvious cytological damage was observed in bone marrow samples taken from the mice inoculated with TS (data not shown). Furthermore, approximately 100% increases in the megakariocyte counts both in the bone marrow and in the spleen were observed 3 days after a single TS injection (10 μg), supporting the hypothesis that the bone marrow was essentially unaltered. These findings were also supported by the recovery of the normal platelet count (Fig. 1A). To further test the effect that the enzyme had on the circulating platelets, lethally irradiated mice were inoculated

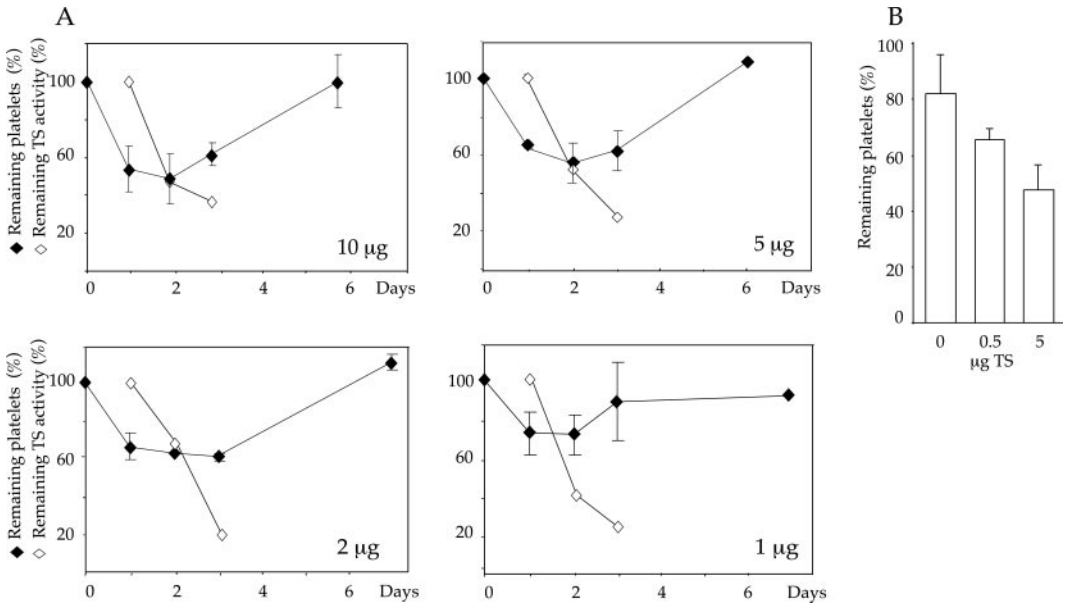

FIG. 1. TS reduces the life span of platelets. (A) Administration of TS induces transient thrombocytopenia. C3H/HeJ mice were inoculated intravenously with different amounts of recombinant TS. The platelet counts and the remaining TS activity in the blood are expressed as percentages. The *P* values for comparisons with data obtained on day 0 or on day 6 or 7 were as follows: for platelet counts on days 1, 2, and 3 in the 10-μg dose group, $P < 0.002$; for platelet counts on days 1, 2, and 3 in the 5-μg dose group, $P < 0.001$; for platelet counts on days 1, 2, and 3 in the 2-μg dose group, $P < 0.001$; and for platelet counts on days 1 and 2 in the 1-μg dose group, $P < 0.05$. (B) TS acts on the circulating platelets. C3H/HeJ mice were lethally irradiated, and then different amounts of TS were given intravenously. Platelet counts were determined 18 h later. For a comparison of the group that received no TS and the group that received 5 μg of TS, the *P* value was <0.01. Each group contained at least three animals.

with a unique dose of TS. These bone marrow-depleted mice exhibited a strong reduction in the platelet count (Fig. 1B), supporting the hypothesis that TS was able to act on platelets in the circulation.

Platelet surface sialic acid content is reduced by TS. Surface sialic acid is critical for the presence of platelets, red blood cells, and glycoproteins in the circulation (23, 35). Therefore, the surface sialic acid content of platelets was quantified after TS treatment to verify the ability of this virulence factor to affect the platelet life span and to produce thrombocytopenia by direct action on the platelets. To do this, platelets were incubated with different amounts of TS (0 to 6 μg) (Fig. 2), and the remaining surface α(2,3)-linked sialic acid was quantified. A significant reduction in the sialic acid content was observed after TS treatment even when as little as 0.5 μg of the enzyme was used (Fig. 2A). To test whether treatment with these amounts of TS could alter the platelet life span, GFP-expressing platelets were assayed. When tracer platelets were treated with 0.5 μg of TS, they were rapidly removed from the circulation after administration into naïve mice (Fig. 2B). The results of these experiments clearly supported the hypothesis that TS is able to remove sialic acid from the cell surface and that this activity is associated with the reduction in the platelet life span.

Leukosialin is not critical for platelet depletion by TS. CD43 (leukosialin, sialophorin) is a heavy sialylated mucin that accounts for about 20 to 25% of the total surface area of white cells and is a substrate of TS (37). Therefore, to analyze its possible involvement, CD43 knockout (KO) mice were assayed. The blood platelet count and the amount of sialic acid in these KO animals were indistinguishable from the values for $CD43^{+/+}$ mice. Administration of TS to KO animals induced thrombocytopenia to an extent that was similar to the extent in $CD43^{+/+}$ mice. The half-life of the $CD43^{-/-}$ platelets was determined by passive transfer of $CD43^{-/-}$ GFP-expressing platelets to both CD43 KO and $CD43^{+/+}$ mice and was found to be similar to that of control $CD43^{+/+}$ GFP-expressing platelets. Since no CD43 was expressed, it seems that the other sialylated glycoconjugates (i.e., glycoproteins, gangliosides) compensated for the total sialic acid content required for the platelets to remain in the blood, and they also were suitable targets for the enzyme.

Kupffer cells are responsible for the clearance of TS-altered platelets from the blood. Beyond the small number of platelets consumed daily to maintain hemostasis, the spleen and liver are the organs where sequestration of damaged, altered, or aged platelets occurs. It has been postulated that the decrement of sialic acid from the surface of the platelets results in recognition of these cells as aged or foreign by the reticuloendothelial system (18, 23). As a first approach to assay the involvement of this compartment in the removal of the TS-altered platelets, splenectomized mice were tested. The animals received a high dose of TS (10 μg), and there was no difference in the reduction of blood platelet counts after injection between naïve and splenectomized mice (compare Fig. 1 and 3A). To further analyze this compartment, we employed GFP-expressing platelets treated with TS and then transfused them to splenectomized and normal mice. In agreement with data shown in Fig. 3A, treated fluorescent platelets were rapidly removed in splenectomized mice, as they were in normal

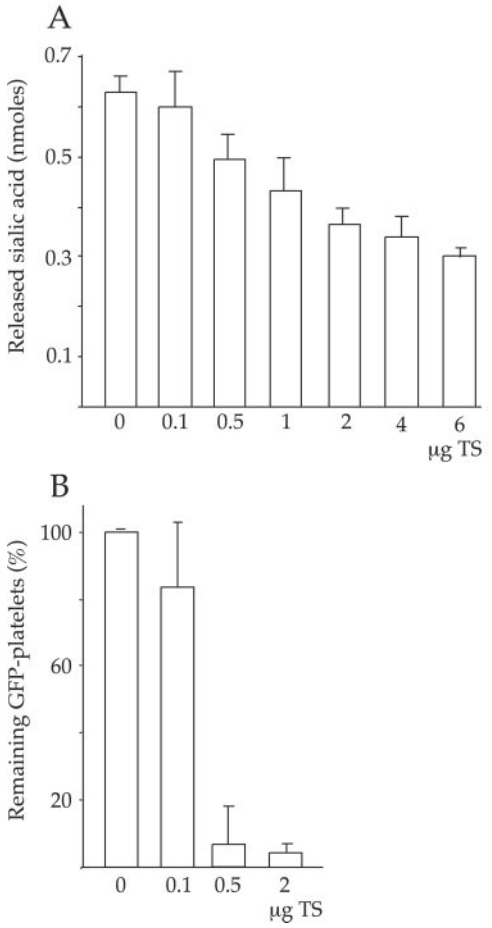

FIG. 2. Platelet desialylation by TS correlates with a reduction in the life span. (A) Platelets (5×10^7 cells) were treated in vitro with different amounts of TS, and the released sialic acid was quantified. For all groups that received 0.5 μg of TS or more, the data were statistically significant (starting at $P < 0.05$ for the 0.5-μg group) for comparisons with the untreated control. (B) GFP-expressing platelets were treated in vitro with different amounts of TS and then transferred to naïve animals. Green platelet counts were determined 18 h later by fluorescence microscopy. The results are expressed as percentages of the remaining green platelets; 100% was defined as the value for controls that received platelets not treated with TS. The values for groups that were treated with 0.5 and 2 μg of TS were found to be statistically significantly different from the other values ($P < 0.005$). Each group contained at least three animals.

receptor animals (Fig. 3B). These results indicated that the spleen is not the main organ for TS-altered platelet removal.

To analyze other components that might be involved, all the professional phagocytes, including Kupffer cells, were depleted. Mice were treated with liposome-encapsulated clodronate, and 24 h later a high dose (10 μg) of TS was administered (4, 22, 41, 42). In spite of the fact that a similar strong treatment induced the loss of a great number of platelets, as shown in Fig. 1A, only a minimal reduction in the platelet count was observed after TS was injected into phagocyte-depleted mice (Fig. 3C). In addition, this small reduction could be explained by the formation and sequestration of platelet-liposome microaggregates (32). Together with the findings obtained with splenectomized mice, these results support the hypothesis that the Kupffer cells play a central role as scavengers for the TS-altered platelets.

TS is associated with the thrombocytopenia observed during *T. cruzi* infection. Thrombocytopenia is described as one of the more relevant hematological findings during early *T. cruzi* infection and correlates well with the detection of TS activity in blood, which indirectly supports the hypothesis that it participates in the platelet count reduction process.

Two main possible effects have to be considered as effects that are involved in the induction of thrombocytopenia during infection, disabling of the bone marrow and alteration of the circulating platelets that leads to their destruction. To determine whether during infection platelets are altered in a way that leads to their clearance, unaltered GFP-expressing platelets were transfused to *T. cruzi*-infected animals. As shown in Fig. 4B, green platelets disappeared from the blood at a higher rate in infected animals than in naïve animals. This finding demonstrated that during infection, the platelet life span is shortened independent of a possible effect of the inflammatory activities derived from the infectious process on the bone marrow.

Recently, it was shown that TS activity is differentially expressed in the main *T. cruzi* lineages and is shed into the milieu at higher rates with *T. cruzi* lineage II than with *T. cruzi* lineage I (33). Circulating TS activity is detected only during the acute phase in mice infected with *T. cruzi* lineage II since in mice infected with *T. cruzi* lineage I strains neutralizing antibodies are developed (33). Therefore, it can be postulated that thrombocytopenia might be related to this differential expression. To test this hypothesis, mice were infected with *T. cruzi* strains that were representatives of the two lineages, and the platelet counts were determined. The K-98 parasites (*T. cruzi* lineage I representative) induced only transient thrombocytopenia, in contrast to the strong platelet depletion observed in the animals infected with Q501/3 (*T. cruzi* lineage II) (Fig. 4A).

To directly test whether TS is actually associated with the circulating platelet depletion in acute Chagas' disease, the enzymatic activity during *T. cruzi* acute infection was neutralized. For this purpose, mice were infected with Q501/3 (a *T. cruzi* lineage II strain) and passively transfused with a unique dose of a TS-neutralizing monoclonal antibody at days 10 to 12 postinfection. One day later the animals were transfused with untreated GFP-expressing platelets. In the infected mice in which the *T. cruzi*-derived TS activity was neutralized, fluorescent platelets were cleared at a rate that was lower than the rate in the mice that received unrelated antibodies (Fig. 4B). These results indicate that the TS activity is directly involved in the thrombocytopenia induced by *T. cruzi* in a mammalian infection by altering the circulating platelets.

DISCUSSION

During experimental infection of mice with *T. cruzi*, strong thrombocytopenia is observed (9, 27), suggesting the possible involvement of TS from the parasite that alters the platelet surface sialic acid content by acting as a neuraminidase (13). From the results reported here we concluded that the capacity of this blood-borne virulence factor to alter the sialylation of the platelet surface directly affects the life span of platelets. It can be postulated that the constant impact of the shed TS, even

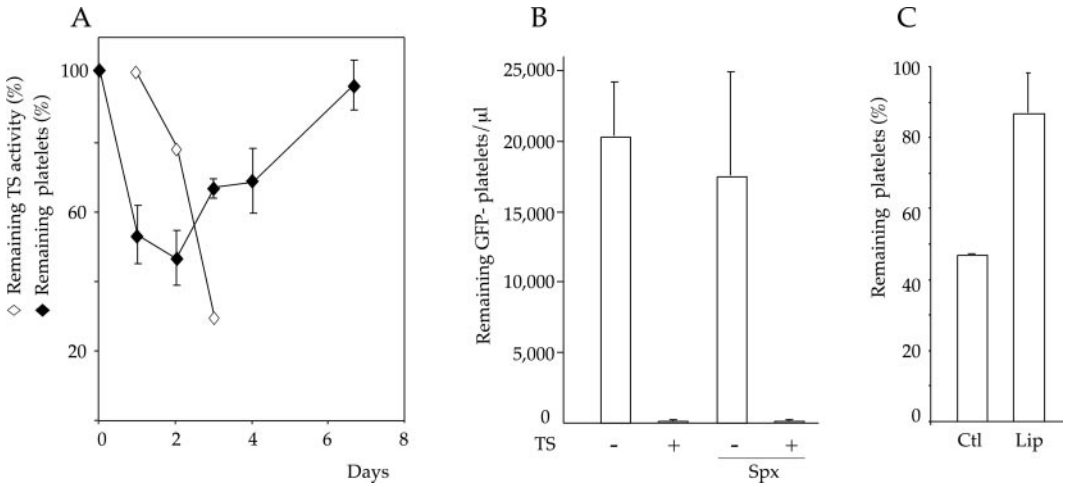

FIG. 3. Kupffer cells are involved in the clearance of TS-altered platelets. (A) Splenectomized BALB/cJ mice were inoculated with 10 μg of TS i.v. The platelet counts and the remaining TS activity in the blood are expressed as percentages. The *P* values for comparisons with data obtained on days 0 and 7 were as follows: for platelet counts on days 1, 2, and 3, $P < 0.001$; and for platelet counts on day 4, $P < 0.05$. (B) GFP-expressing platelets were transferred after in vitro TS treatment (2 μg) to either normal or splenectomized (Spx) animals. Green platelet counts were determined 18 h later by fluorescence microscopy. For a comparison of the group that received TS and the group that did not receive TS, the *P* value was <0.005. (C) Mice were treated with liposome-encapsulated clodronate 24 h before administration of TS (10 μg). Platelet counts determined 18 h after TS administration are expressed as percentages of the value for naïve animals. Ctl, control animals that received TS; Lip, liposome-treated animals that received TS. For a comparison of the control animals and the liposome-treated animals, the *P* value was <0.01. Each group contained at least four animals.

in small amounts, on the platelets finally leads to the development of the thrombocytopenia observed in the animals. However, during the infection, other molecules, either molecules from the parasite or molecules derived from the immune response against the infection, like cytokines or other inflammatory products, might also collaborate in this process. Nevertheless, clearance of transferred platelets was prevented in infected animals by passive transfer of a monoclonal neutralizing antibody, which provided strong evidence that TS is involved in the thrombocytopenia that is induced. Platelets were postulated to be capable of inducing parasite lysis after interaction via their C3b receptor (38), and a direct role for platelets in in vivo parasite clearance (but only in the presence of antibodies) was also suggested (36, 39). Based on this line of evidence, TS might participate in prevention of parasite elimination during the acute phase of the infection by reducing the amount of platelets or by altering the platelets that interact with the parasite.

The bone marrow becomes depleted during acute infection with virulent *T. cruzi* strains (27). Although a possible effect of prolonged exposure to TS, as happens during infection, cannot be disregarded, from the evidence at hand it seems that the destruction of this tissue is more likely to be due to the immune response or to other parasite-derived factors. No obvious evidence of morphological alterations of the bone marrow cells was found after a single injection of the enzyme, and the thrombopoiesis capacity remained unaltered, as suggested by the recovery of normal platelet counts when the TS activity disappeared. This is consistent with the finding that in surviving animals the normal platelet count recovers when the parasitemia is controlled (9). Thrombopoietin is constitutively produced by the liver and kidneys, but its actual activity with precursor cells is regulated by the number of circulating platelets because of the expression of the corresponding receptor (c-Mpl) on the surface, which tunes the activity (20, 43). Therefore, the observed increase in the number of megakariocytes in both the bone marrow and the spleen after TS injection seems to accompany the thrombocytopenia induced by the platelet destruction instead of being related to a possible direct effect of the TS on the earlier precursor cells.

TS is in fact a modified sialidase that has acquired the ability to transfer the sialyl residue to a β-linked terminal galactose instead to water (hydrolysis) (7, 8). Although a possible role in redistribution of the sialyl residues among the surface glycoconjugates cannot be disregarded, the loss of sialyl residues from the platelets after TS treatment strongly supports the hypothesis that their increased clearance is due to the α(2,3)-neuraminidase activity of the enzyme. As shown in Fig. 2, depletion of α(2,3)-linked sialic acid occurs at a level that is able to alter the life span of platelets in blood (18). It should be stressed that the sialyl residues linked by α(2,3) linkages, but not the residues linked by α(2,6) linkages, are the residues that are critical in determining the half-life of platelets and seric factors (15) and that the α(2,3) linkage is the linkage with specificity or a preferred configuration for almost all of the pathogen-derived sialidases, suggesting a general target for these virulence factors.

Desialylation is known to lead to sequestration of platelets from the blood by the reticuloendothelial system. Here we determined that the spleen is not critical in the destruction of TS-altered platelets, and our findings strongly support involvement of the Kupffer cells in this process instead. Similar experimental approaches recently resulted in determination of the involvement of Kupffer cells in the clearance of erythrocytes deficient in glycosyl phosphatidylinositol-linked proteins (22). In this regard it should be pointed out that strong thrombocytopenia also occurs during infection of splenectomized mice that show progressive platelet depletion, as well as in

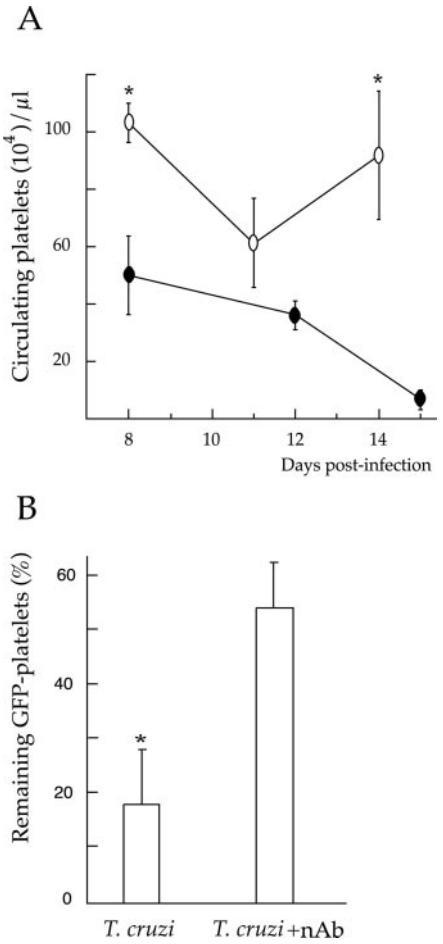

FIG. 4. TS is associated with thrombocytopenia during *T. cruzi* infection. (A) Platelet counts in *T. cruzi*-infected mice. Mice were infected with either 50,000 K-98 parasite (lineage I clone) blood trypomastigotes (○) or 250 Q501/3 parasite (lineage II clone) blood trypomastigotes (●). An asterisk indicates that the *P* value is at least <0.01. There were five animals in each group. (B) In three separate experiments, GFP-expressing platelets were transferred to lethal *T. cruzi* strain-infected C3H/HeJ animals at day 11, 12, or 13 postinfection. Recipients were passively transfused with TS-neutralizing monoclonal antibody (nAb) 24 h before green platelet transfusion. The fluorescent platelet count was determined 18 h later. The green platelet count in transfused uninfected mice was defined as 100%. The asterisk indicates that the *P* value is <0.01. Each group contained at least three animals.

infected control animals (9). The fact that a parasite-derived virulence factor is involved is further supported by our findings obtained with parasites that were representative of the main *T. cruzi* lineages (Fig. 4), which showed that a nonlethal strain (*T. cruzi* lineage I) that expressed or shed smaller amounts of TS was unable to induce persistent thrombocytopenia, although comparable levels of parasitemia were reached. Together with the TS neutralization assay results, these results indicate that TS is the virulence factor that causes the thrombocytopenia during the acute phase of Chagas' disease.

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REFERENCES

1. **Aakhus, A. M., P. Stavem, T. Hovig, T. M. Pedersen, and N. O. Solum.** 1990. Studies on a patient with thrombocytopenia, giant platelets and a platelet membrane glycoprotein Ib with reduced amount of sialic acid. Br. J. Haematol. **74:**320–329.
2. **Agusti, R., A. S. Couto, O. Campetella, A. C. Frasch, and R. M. de Lederkremer.** 1998. Structure of the glycosylphosphatidylinositol-anchor of the trans-sialidase from *Trypanosoma cruzi* metacyclic trypomastigote forms. Mol. Biochem. Parasitol. **97:**123–131.
3. **Agusti, R., A. S. Couto, O. E. Campetella, A. C. Frasch, and R. M. de Lederkremer.** 1997. The *trans*-sialidase of *Trypanosoma cruzi* is anchored by two different lipids. Glycobiology **7:**731–735.
4. **Alves-Rosa, F., C. Stanganelli, J. Cabrera, N. van Rooijen, M. S. Palermo, and M. A. Isturiz.** 2000. Treatment with liposome-encapsulated clodronate as a new strategic approach in the management of immune thrombocytopenic purpura in a mouse model. Blood **96:**2834–2840.
5. **Buscaglia, C. A., J. Alfonso, O. Campetella, and A. C. Frasch.** 1999. Tandem amino acid repeats from *Trypanosoma cruzi* shed antigens increase the half-life of proteins in blood. Blood **93:**2025–2032.
6. **Buscaglia, C. A., O. Campetella, M. S. Leguizamon, and A. C. Frasch.** 1998. The repetitive domain of *Trypanosoma cruzi trans*-sialidase enhances the immune response against the catalytic domain. J. Infect. Dis. **177:**431–436.
7. **Buschiazzo, A., A. C. Frasch, and O. Campetella.** 1996. Medium scale production and purification to homogeneity of a recombinant *trans*-sialidase from *Trypanosoma cruzi*. Cell. Mol. Biol. (Noisy-le-Grand) **42:**703–710.
8. **Buschiazzo, A., G. A. Tavares, O. Campetella, S. Spinelli, M. L. Cremona, G. Paris, M. F. Amaya, A. C. Frasch, and P. M. Alzari.** 2000. Structural basis of sialyltransferase activity in trypanosomal sialidases. EMBO J. **19:**16–24.
9. **Cardoso, J. E., and Z. Brener.** 1980. Hematological changes in mice experimentally infected with *Trypanosoma cruzi*. Mem. Inst. Oswaldo Cruz **75:**97–104.
10. **Choi, S. I., J. V. Simone, and L. J. Jorney.** 1972. Neuraminidase-induced thrombocytopenia in rats. Br. J. Haematol. **22:**93–101.
11. **Clezardin, P., J. L. McGregor, M. Dechavanne, and K. J. Clemetson.** 1985. Platelet membrane glycoprotein abnormalities in patients with myeloproliferative disorders and secondary thrombocytosis. Br. J. Haematol. **60:**331–344.
12. **Cremona, M. L., D. O. Sanchez, A. C. Frasch, and O. Campetella.** 1995. A single tyrosine differentiates active and inactive *Trypanosoma cruzi trans*-sialidases. Gene **160:**123–128.
13. **Csete, M., B. I. Lev, and M. E. Pereira.** 1985. An influenza virus model for *Trypanosoma cruzi* infection: interactive roles for neuraminidase and lectin. Curr. Top. Microbiol. Immunol. **117:**153–165.
14. **de Titto, E. H., and F. G. Araujo.** 1988. Serum neuraminidase activity and hematological alterations in acute human Chagas' disease. Clin. Immunol. Immunopathol. **46:**157–161.
15. **Ellies, L. G., D. Ditto, G. G. Levy, M. Wahrenbrock, D. Ginsburg, A. Varki, D. T. Le, and J. D. Marth.** 2002. Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. Proc. Natl. Acad. Sci. USA **99:**10042–10047.
16. **Frasch, A. C.** 2000. Functional diversity in the *trans*-sialidase and mucin families in *Trypanosoma cruzi*. Parasitol. Today **16:**282–286.
17. **Goswami, K., and B. C. Koner.** 2002. Level of sialic acid residues in platelet proteins in diabetes, aging, and Hodgkin's lymphoma: a potential role of free radicals in desialylation. Biochem. Biophys. Res. Commun. **297:**502–505.
18. **Greenberg, J., M. A. Packham, J. P. Cazenave, H. J. Reimers, and J. F. Mustard.** 1975. Effects on platelet function of removal of platelet sialic acid by neuraminidase. Lab. Investig. **32:**476–484.
19. **Grottum, K. A., and M. Jeremic.** 1973. Neuraminidase injections in rabbits. Reduced platelet surface charge, aggregation and thrombocytopenia. Thromb. Diath. Haemorrh. **29:**461–469.
20. **Harker, L. A., L. K. Roskos, U. M. Marzec, R. A. Carter, J. K. Cherry, B. Sundell, E. N. Cheung, D. Terry, and W. Sheridan.** 2000. Effects of megakaryocyte growth and development factor on platelet production, platelet life span, and platelet function in healthy human volunteers. Blood **95:**2514–2522.
21. **Hoffman, M., D. M. Monroe, and H. R. Roberts.** 1992. A rapid method to isolate platelets from human blood by density gradient centrifugation. Am. J. Clin. Pathol. **98:**531–583.

22. **Jasinski, M., P. Pantazopoulos, R. P. Rother, N. Van Rooijen, W. C. Song, H. Molina, and M. Bessler.** 2004. A novel mechanism of complement-independent clearance of red cells deficient in glycosyl phosphatidylinositol-linked proteins. Blood **103:**2827–2834.
23. **Kotze, H. F., V. van Wyk, P. N. Badenhorst, A. D. Heyns, J. P. Roodt, and M. G. Lotter.** 1993. Influence of platelet membrane sialic acid and platelet-associated IgG on ageing and sequestration of blood platelets in baboons. Thromb. Haemostasis **70:**676–680.
24. **Leguizamón, M. S., O. Campetella, G. Russomando, M. Almiron, I. Guillen, S. M. Ganzález Cappa, and A. C. Frasch.** 1994. Antibodies inhibiting *Trypanosoma cruzi trans*-sialidase activity in sera from human infections. J. Infect. Dis. **170:**1570–1574.
25. **Leguizamón, M. S., E. Mocetti, H. Garcia Rivello, P. Argibay, and O. Campetella.** 1999. Trans-sialidase from *Trypanosoma cruzi* induces apoptosis in cells from the immune system *in vivo*. J. Infect. Dis. **180:**1398–1402.
26. **Leguizamón, M. S., G. Russomando, A. Luquetti, A. Rassi, M. Almiron, S. M. González-Cappa, A. C. Frasch, and O. Campetella.** 1997. Long-lasting antibodies detected by a *trans*-sialidase inhibition assay of sera from parasite-free, serologically cured chagasic patients. J. Infect. Dis. **175:**1272–1275.
27. **Marcondes, M. C., P. Borelli, N. Yoshida, and M. Russo.** 2000. Acute *Trypanosoma cruzi* infection is associated with anemia, thrombocytopenia, leukopenia, and bone marrow hypoplasia: reversal by nifurtimox treatment. Microbes Infect. **2:**347–352.
28. **McGregor, J. L., K. J. Clemetson, E. James, E. F. Luscher, and M. Dechavanne.** 1980. A comparison of the major platelet membrane glycoproteins from Bernard-Soulier syndrome with normals after radiolabelling of sialic acid or terminal galactose/N-acetylgalactosamine residues. Thromb. Res. **17:**713–718.
29. **Mucci, J., A. Hidalgo, E. Mocetti, P. F. Argibay, M. S. Leguizamón, and O. Campetella.** 2002. Thymocyte depletion in *Trypanosoma cruzi* infection is mediated by *trans*-sialidase-induced apoptosis on nurse cell complex. Proc. Natl. Acad. Sci. USA **99:**3896–3901.
30. **Pereira, M. E.** 1983. A developmentally regulated neuraminidase activity in *Trypanosoma cruzi*. Science **219:**1444–1446.
31. **Powell, L. D., and G. W. Hart.** 1986. Quantitation of picomole levels of N-acetyl- and N-glycolylneuraminic acids by a HPLC-adaptation of the thiobarbituric acid assay. Anal. Biochem. **157:**179–185.
32. **Reinish, L. W., M. B. Bally, H. C. Loughrey, and P. R. Cullis.** 1988. Interactions of liposomes and platelets. Thromb. Haemostasis **60:**518–523.
33. **Risso, M. G., G. B. Garbarino, E. Mocetti, O. Campetella, S. M. González Cappa, C. A. Buscaglia, and M. S. Leguizamón.** 2004. Differential expression of a virulence factor, the *trans*-sialidase, by the main *Trypanosoma cruzi* phylogenetic lineages. J. Infect. Dis. **189:**2250–2259.
34. **Schenkman, S., D. Eichinger, M. E. Pereira, and V. Nussenzweig.** 1994. Structural and functional properties of *Trypanosoma trans*-sialidase. Annu. Rev. Microbiol. **48:**499–523.
35. **Steiner, M., and S. Vancura.** 1985. Asymmetrical loss of sialic acid from membrane glycoproteins during platelet aging. Thromb. Res. **40:**465–471.
36. **Takehara, H. A., and I. Mota.** 1991. The possible mechanism of action of IgG antibodies and platelets protecting against *Trypanosoma cruzi* infection. Braz. J. Med. Biol. Res. **24:**759–765.
37. **Todeschini, A. R., M. P. Nunes, R. S. Pires, M. F. Lopes, J. O. Previato, L. Mendonca-Previato, and G. A. DosReis.** 2002. Costimulation of host T lymphocytes by a trypanosomal *trans*-sialidase: involvement of CD43 signaling. J. Immunol. **168:**5192–5198.
38. **Umekita, L. F., and I. Mota.** 1989. In-vitro lysis of sensitized *Trypanosoma cruzi* by platelets: role of C3b receptors. Parasite Immunol. **11:**561–566.
39. **Umekita, L. F., and I. Mota.** 1990. Role of platelets in the in vivo removal of *T. cruzi* from circulation. Braz. J. Med. Biol. Res. **23:**593–596.
40. **Urbina, J. A., and R. Docampo.** 2003. Specific chemotherapy of Chagas disease: controversies and advances. Trends Parasitol. **19:**495–501.
41. **Van Rooijen, N., and A. Sanders.** 1996. Kupffer cell depletion by liposome-delivered drugs: comparative activity of intracellular clodronate, propamidine, and ethylenediaminetetraacetic acid. Hepatology **23:**1239–1243.
42. **Van Rooijen, N., and A. Sanders.** 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Methods **174:**83–93.
43. **Wendling, F.** 1999. Thrombopoietin: its role from early hematopoiesis to platelet production. Haematologica **84:**158–166.

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