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

María Virginia Tribulatti,1 Juan Mucci,1 Nico Van Rooijen,2 María Susana Leguizamón,3 and Oscar Campetella1*

Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín,1 and Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires,2 Buenos Aires, Argentina, and Department of Molecular Cell Biology and Immunology, Faculty of Medicine, Free University, Amsterdam, The Netherlands3

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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. Plasmatid 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+...
CD3+ thymocyte deletion 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 platelet 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/c, C3H/HeJ, and C57BL/6J mouse strains and green fluorescent protein (GFP)-expressing C57BL/6-Tg(ActbEGFP)OsbJ hemizygous transgenic male mice were originally obtained from The Jackson Labs (Bar Harbor, Maine) and were bred in our facilities. B6.CD43−/− female mice backcrossed eight times to C57BL/6 were generously provided by Anne I. Sperling (University of Chicago) and crossed with GFP-expressing males. F1 CD43−/− GFP-expressing mice 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 Tyr342His point mutation (12) were expressed in E. coli (12) and purified with immobilized metal affinity chromatography through Ni2+−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 saline.

**TS assay.** The enzymatic activity of purified TS was assayed by measuring the amount of sialic acid residue transferred from sialyllactose (Sigma) to [14C]lactose (31). 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 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.

**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 megakaryocyte 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

and incubated with an excess (6 μM) 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 2 μg of TS. Platelet counts were determined 18 h later.

**Determination of in vivo survival of TS-treated platelets.** GFP-expressing platelets (5 × 107 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. Phosphatidylycholine (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. Pitovcys R. Muia, 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 × 10⁵ to 8.5 × 10⁵ parasites/ml, and 24 h later 1 × 10⁵ to 2 × 10⁵ 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).

**Statistical analysis.** Student’s t test was employed.
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 decrease 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

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**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.
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 naive 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
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 megakaryocytes 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

![Graph A](a.png)  ![Graph B](b.png)  ![Graph C](c.png)
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 1) 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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