Trypanosoma cruzi trans-Sialidase: Enhancement of Virulence in a Murine Model of Chagas' Disease

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Summary

Trypanosoma cruzi, the etiological agent of Chagas' disease, expresses a *trans*-sialidase at highest levels in infective trypomastigotes, where it attaches to the plasma membrane by a glycophosphoinositol linkage. Bound enzyme sheds into the extracellular milieu in a soluble form. Experiments performed in vitro suggest that the *trans*-sialidase participates in several parameters of *T. cruzi*–host interactions, like cell adhesion and complement resistance. However, the role that membrane-bound and soluble *trans*-sialidase plays in the infection of mammals is not understood. To begin to study the role the enzyme may play in vivo, *T. cruzi* trypomastigotes were inoculated subcutaneously into mice that had been sensitized for various times with the purified protein. A single dose of either endogenous or recombinant *trans*-sialidase injected into the connective tissues of BALB/c mice greatly enhanced parasitemia and mortality. Maximum enhancement was achieved with 1–2-h priming. Injection of the enzyme after the parasites had been established in the inoculation site had little, if any, consequence in modifying virulence. The enhancement did not seem to be through a direct effect of the enzyme on trypomastigote–host cell interactions because it occurred when the sites of *trans*-sialidase sensitization and parasite inoculation were physically separate. Rather, virulence enhancement seemed to depend on inflammatory cells, since priming with *trans*-sialidase had no significant effect in severe combined immunodeficiency mice, which lack functional T and B lymphocytes. However, antibody response to *T. cruzi* in the *trans*-sialidase–primed BALB/c mice was the same as in the control animals. Virulence enhancement was specific for the *trans*-sialidase because it did not occur in mice primed with Newcastle virus sialidase, which has the same substrate specificity as the *T. cruzi* enzyme, or with the sialidase from the bacterium *Vibrio cholerae*, whose substrate specificity is broader than the trypanosome sialidase. Furthermore, no enhancement of virulence occurred after sensitization with another adhesion protein (penetrin) purified from *T. cruzi* trypomastigotes and engineered bacteria, nor with bacterial lipopolysaccharide. The virulence-promoting activity of soluble *trans*-sialidase in the mouse model may be physiologically relevant because it was achieved with tiny doses, ~1–2 μg/kg, raising the possibility that neutralization of the enzyme with specific probes could impair the development of Chagas' disease. In fact, a monoclonal antibody specific for the tandem repeat in the *trans*-sialidase COOH terminus enhanced infection of BALB/c mice, in agreement with earlier experiments in vitro, whereas antibodies against an amino acid sequence in the Cys region had the opposite effect.

Trypanosoma cruzi causes Chagas' disease, an incurable debilitating condition that affects millions of people in Latin America (1–3). Acute disease lasts several months, and it is characterized by parasitemia caused by rapid intracellular replication of *T. cruzi* in various tissues of the host. In contrast, there is little replication of the parasites in chronic Chagas' disease, which develops several decades after the initial infection and is characterized by the megasyndrome, particularly cardiomegaly, megaesophagus, and megacolon, which generally leads to death. The molecular mechanisms underlying the pathogenesis of Chagas' disease remain unknown.

T. cruzi expresses a developmentally regulated *trans*-sialidase (4), an enzyme that hydrolyzes sialic acid from glycoconjugates, releasing the carbohydrate into water (sialidase or neuraminidase activity) (5) or transferring it to suitable βGal acceptors (transferase activity) (6, 7). The sialidase and transferase activities are both specific for sialic acid in α2,3 linkage (8, 9). Therefore, the *trans*-sialidase produces asialoglycoconjugates from sialyl donor substrates, and at the same time generates α2,3-linked sialyl derivatives of βGal acceptor substrates.

The *trans*-sialidase is located on the surface of infective trypomastigotes (10) by a glycophosphoinositol anchor (11–13), and for this reason, it is readily shed into the environment whether the parasites are in culture supernatants (14), in the

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cytosol of infected cells (15), or in the circulation of Chagasic patients (16). Thus, in theory, the *trans*-sialidase could function in host–parasite interactions either on the surface of trypomastigotes or as a soluble mediator (3).

Surface membrane *trans*-sialidase is believed to promote trypomastigote adhesion by reacting with α2,3-linked sialyl epitopes present on the plasma membrane of host cells (17–19). Consequently, *T. cruzi* attached to sialic acid–deficient epithelial cells to a lesser extent than to sialic acid–containing parental cells (18–20). Resialylation of sialic acid–deficient cell lines restored *T. cruzi* attachment to levels similar to those of wild-type cells (17, 19). Furthermore, purified *trans*-sialidase at relatively low concentrations (micrograms per milliliter range) competitively blocked *T. cruzi* binding to wild-type epithelial host cells (17). The function of the *trans*-sialidase as an adhesion molecule is explained by its low intrinsic sialidase activity (~2–5% of the sialyltransferase activity), such that in the absence of βGal acceptors, the enzyme should bind to sialoglycoconjugates in a lectin type of action (4, 9).

The *trans*-sialidase may have other roles in *T. cruzi*–host interactions in addition to mediation of attachment. For example, the enzyme has been postulated to participate in the escape of trypomastigotes from phagolysosomes into the cytosol (13), as well as in the resistance of trypomastigotes to complement activation (4). Interestingly, when the trypomastigotes exit host cells into the extracellular environment, they rapidly acquire sialic acid residues by the catalytic action of the *trans*-sialidase, which may confer infective properties to the parasite (7).

These inferences about the role of the *trans*-sialidase in adhesion and other parameters of host–*T. cruzi* interactions were all based on assays performed in vitro. To determine the possible significance of the *trans*-sialidase in an in vivo situation, we followed the course of infection of Chagasic mice sensitized with purified *trans*-sialidase and with *trans*-sialidase antibodies before parasite challenge. BALB/c and C3H mice primed with extremely low amounts of the *trans*-sialidase (~20–30 ng per mouse) became remarkably sensitive hosts for *T. cruzi* growth. In fact, it was possible to turn a nonlethal dose of *T. cruzi* into a mortal inoculum by sensitizing connective tissues of mice with picomole amounts of the enzyme. A monoclonal antibody reactive with the tandem repeat in the COOH-terminal domain of the *trans*-sialidase enhanced virulence, whereas antibodies against a peptide in the NH_2 terminus were protective. The molecular basis for this virulence-enhancing effect of the *trans*-sialidase remains to be determined, but it appears to depend on functional nonimmune host lymphocytes.

Materials and Methods

Parasites. All studies were performed with the cloned Silvio X-10/4 and MV-13 strains of *T. cruzi* used in earlier studies (14, 20), as well as with Tulahuen clone C2 provided by Dr. Steve Reed (Infectious Diseases Institute, Seattle, WA) (21). Trypomastigotes were propagated in Vero cells as previously described (14, 20).

Antibodies. mAb TCN-2 (IgG_1) was isolated from a C3H mouse chronically infected with a clone of MV-13 that expresses high *trans*-sialidase activity (20). The antibody was purified from hybridoma supernatants on protein G–Sepharose columns (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS, pH 7.5; bound antibodies were eluted with 0.1 M glycine–HCl buffer, pH 2.5, and immediately dialyzed against PBS. Isotype-matched control antibody was a hybridoma against *p*-azophenylarsonate (20), which was also purified on protein G–Sepharose as above. Fab fragments of the antibodies were obtained by digestion with papain as described (22). Sizes of Fab fragments were confirmed by sieving chromatography on Sephadex G-100 and by the absence of binding to protein G–Sepharose.

Antibodies to peptide C (RLPKRMGGSYRC, ref. 12) were elicited in BALB/c or C3H mice by intraperitoneal injection of 100 μg of the synthetic peptide (without a protein carrier) in the presence of complete Freund's adjuvant, followed by three to five injections of the same amount of the synthetic peptide in incomplete Freund's adjuvant every 7–10 d. Anti-peptide C IgG was purified on protein G–Sepharose, and the IgG fraction specific for the synthetic peptide was isolated on an affinity adsorbent prepared by coupling peptide C to Sepharose 4B by activation with cyanogen bromide (~3–5 mg peptide/ml settled beads). IgG (~5 mg) bound to the peptide C–Sepharose column was specifically eluted by addition of soluble synthetic peptide (2 mg/ml) to the equilibrating PBS buffer (yield of anti-peptide C IgG, ~2.3 mg). Peptide C was separated from the antibody by extensive dialysis. Anti-peptide C antibody was also purified from Chagasic sera, which consisted of a pool of sera with indeterminate and chronic Chagas' disease from the endemic area of Bahia, Brazil. These sera are the same pool used in previous studies (14, 23) and were originally prepared by Dr. Rodney Hoff (National Institutes of Health, Bethesda, MD). Peptide C antibodies were purified from Chagasic sera by the same procedure used to isolate mouse anti-peptide antibody.

Purification of trans-Sialidase and Penetrin. *T. cruzi trans*-sialidase was purified as previously described (9). Briefly, conditioned supernatants of Vero cell cultures infected with *T. cruzi* Silvio-X10/4, MV-13, or Tulahuen, were applied to affinity columns of mAb TCN-2 adsorbed to protein G–Sepharose; *trans*-sialidase bound to insolubilized TCN-2 was eluted with synthetic peptide TR (DSSAHGTPSTPA, 10 mg/ml) and separated from the synthetic peptide by fast protein liquid chromatography on a Superose 12 column equilibrated with PBS. The purified protein migrated as a 200-kD doublet as assessed by SDS-PAGE and silver staining (9). The recovery of the isolated enzyme ranged from 5–10 μg/100 ml of conditioned medium. *trans*-Sialidase purified from the Y strain of *T. cruzi* involved Con A affinity binding, sizing, and ion-exchange chromatography (24) and was a gift from Drs. Lain de Carvalho and Victor Nussenzweig (New York University, New York, NY).

Water used in the *trans*-sialidase purification was sterile and pyrogen free (Abbott Laboratories, Abbot Park, IL). The LPS content of some *trans*-sialidase preparations was quantitated by the *Limulus* amebocyte assay following the recommendations of the manufacturer (Associates of Cape Cod Inc., Woods Hole, MA), and it was found to be below the detection limit of the assay.

Recombinant *trans*-sialidase was purified using the same protocol as for the endogenous enzyme. *Escherichia coli* XL-1 blue transformed with Bluescript plasmid containing *trans*-sialidase inserts (7F, 19Y, and 17D) were grown and harvested as previously described (12). The *trans*-sialidase gene in these inserts is on the T7 side of the Bluescript plasmid and is not induced by isopropylthioglycoside. Instead, the *trans*-sialidase protein is expressed constitutively in the cytosol of these bacteria in a soluble form. The yield of affinity-purified recombinant protein ranged from 5 to 50 mg/liter of bacterial culture. Large batches of bacteria were grown in the GRASP

Core Center of the New England Medical Center by Dr. Anne Kane.

Penetrin was isolated from *T. cruzi* trypomastigotes and *E. coli* HBP-6 by heparin–Sepharose chromatography as previously described (25).

Immunoblots. SDS-PAGE and immunoblots of lysates (~10 μg per lane) and purified proteins (0.5 μg per lane) were performed as previously described (20).

Protein Determination. *trans*-Sialidase concentration was estimated by SDS-PAGE. A standard curve was set up by applying various amounts (0.005–0.1 μg per lane) of BSA on a 7.5% SDS–polyacrylamide gel. Proteins in the gel were stained with silver or problue (Integrated Separation Systems, Hyde Park, MA) and quantitated in a laser-scanning densitometer (LKB Instruments, Inc., Gaithersburg, MD). Various volumes of the purified *trans*-sialidase solution were applied to the SDS–polyacrylamide gel in parallel with BSA, and the concentration of the enzyme was estimated by comparison with the BSA standard curve.

Penetrin was estimated by the Bradford procedure following the recommendations of the manufacturer (Bio-Rad Laboratories, Richmond, CA).

trans-Sialidase Assay. The *trans*-sialidase assay was performed as described (9). Enzyme in 10 μl was added to 40 μl of 50 mM PBS, pH 7.2, containing 0.5 mmol of 2,3-sialyllactose (Sigma Chemical Co., St. Louis, MO), 0.25 mmol of [^{14}C]N-acetyllactosamine (4 × 10^5 dpm), 50 μg BSA, and 0.02% NaN_3. After incubation for various times at room temperature, the reaction mixture was diluted to 1.0 ml with distilled water and applied to a column containing 1.0 ml Q-Sepharose equilibrated with water. Sialylated product was eluted with 1 M NaCl and quantitated by scintillation counting.

ELISA. ELISA was a modification of a procedure described earlier (26). In brief, polyvinyl microtiter plates (Corning Glass Inc., Corning, NY) were coated overnight at 4°C with ~100 μg/ml of *T. cruzi* antigens prepared by lysing trypomastigotes (10^8/ml) with the detergent 3-([3-cholamidopropyl]-dimethylammonio)-1-propane-sulfonate (0.1%) containing protease inhibitors (5 mM EDTA, 5 mM iodoacetamide, 5 μg/ml each leupeptin, pepstatin, and soybean trypsin inhibitor). Plates were washed twice with PBS–0.05% Tween 20 and blocked with 1% heat-treated (56°C, 30 min) BSA (room temperature for 1 h), followed by addition of various dilutions of sera of infected and control noninfected mice. After 1 h at room temperature, plates were washed three times with PBS–Tween 20, incubated for 1 h with an alkaline phosphatase–labeled goat anti–mouse antibody (Promega Corp., Madison, WI), and after washing, bound antibody was detected with the substrate *p*-nitrophenyl phosphate (1 mg/ml) in diethanolamine buffer (pH 9.8). Reaction was stopped after 1 h with 3 M NaOH and the absorbance measured at 405 nm using an automated ELISA reader (Bio-Tek Instruments Inc., Winooski, VT).

Chagasic Mouse Model. Female BALB/c and C3H mice (Taconic Farms Inc., Germantown, NY), 8–10 wk old, were injected subcutaneously into the footpads with 12 μl of sterile, pyrogen-free PBS (in tuberculin syringes) without (control) or with purified *trans*-sialidase (1–5 μg/ml), Newcastle virus sialidase (10 mU/ml) (Genzyme Corp., Cambridge, MA), *Vibrio cholerae* sialidase (10 mU/ml) (Calbiochem Corp., La Jolla, CA), penetrin (10–100 μg/ml), and *E. coli* LPS (10 μg/ml) (serotype N0127:B8; Sigma Chemical Co.). After various times, 12 μl of a trypomastigote suspension of the Tulahuen strain (0.2–2 × 10^5/ml), freshly harvested from Vero cell monolayers, was inoculated into the footpad that had been primed with the *trans*-sialidase, or the other footpad, or the peritoneal cavity. In some experiments, *trans*-sialidase priming and parasite inoculation were both done in the two footpads (see Table 1); in this case, the injection consisted of 25 μl (12.5 μl per footpad) of priming solution and parasite suspension. In some experiments, parasites were inoculated in the soft tissue first, followed at different time intervals by *trans*-sialidase. Mice were removed from the study if extensive bleeding occurred after the subcutaneous injection.

Mice were bled in the tail at various times after inoculation, and bloodstream trypomastigotes were quantitated by two methods: (*a*) counting in a hemocytometer under a light microscope, as described by Brener (27), and (*b*) PCR of the blood parasites using a highly sensitive probe, as described by Moser et al. (28). Results given in the various figures described below were obtained with the first method described above.

Results

Virulence-promoting Activity of the T. cruzi trans-Sialidase. One of the pathways used by *T. cruzi* to attach to cultured mammalian cells depends on host cell receptors containing α2,3-linked sialic acid epitopes (3, 17). In an attempt to test whether *T. cruzi* uses sialyl residue receptors during invasion in vivo, we primed the footpads of BALB/c mice with small amounts of the purified *T. cruzi trans*-sialidase. By analogy with results obtained in vitro (17), the expectation was that priming soft tissues with the purified *trans*-sialidase, followed by a *T. cruzi* inoculum, would reduce parasite invasion as measured by parasitemia and survival of the mouse host.

But contrary to expectation, mice sensitized with the *trans*-sialidase exhibited high parasitemia and died early compared with control mice, which were treated in the same way as the experimental mice, except that priming of the tissues was with vehicle medium without the sialidase. In fact, using an inoculum of trypomastigotes that produces transient parasitemia, it was possible to turn a nonlethal dose of *T. cruzi* into a mortal one by priming soft tissues of mice with a single amount of enzyme as small as 20 ng per mouse (Fig. 1, *A* and *C*). This enhancement of virulence was obtained with *trans*-sialidase isolated from the Tulahuen strain of *T. cruzi*. *trans*-Sialidase purified from another strain of the parasite, Silvio X-10/4, likewise enhanced *T. cruzi* virulence (see below).

In addition to the Tulahuen and Silvio X-10/4 *trans*-sialidases isolated in our laboratory, we tested Y strain *trans*-sialidase purified in a different laboratory (24) by a protocol distinct from ours (see Materials and Methods). The Y strain enzyme, as did the other *trans*-sialidases, potently enhanced parasitemia and mortality of BALB/c mice (Fig. 1, *B* and *D*, respectively).

The virulence-promoting activity of the *trans*-sialidase was not confined to BALB/c mice (H-2^d haplotype) since the enzyme also enhanced parasitemia and mortality of C3H mice infected with *T. cruzi* (H-2^k haplotype) (data not shown).

Virulence-promoting Activity by Recombinant trans-Sialidase. The *trans*-sialidase used in the experiments described above was isolated from conditioned media of monolayers infected with *T. cruzi*. Although the *T. cruzi* enzyme was homogeneously pure, the possibility existed that the virulence-promoting activity of the preparations was caused by contaminants derived from fetal calf sera and/or from secretory products of the fibroblast monolayers. To discard this possibility, we used recombinant *trans*-sialidase purified from bacteria transformed with plasmids 7F, 19Y, and 17D, which contain the *trans*-

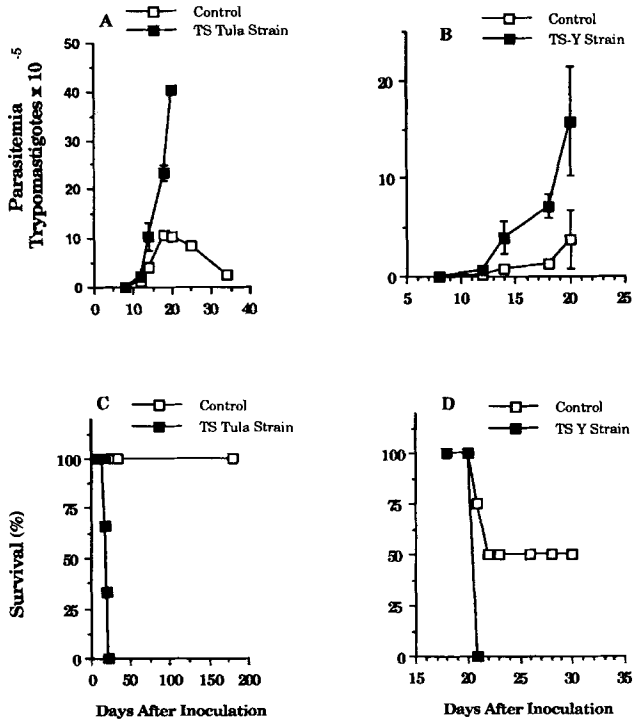

Figure 1. Enhancement of *T. cruzi* virulence for BALB/c mice sensitized with the *trans*-sialidases. Groups of four mice were sensitized in the soft tissue of their footpads with ~20 ng of *trans*-sialidase purified from Tulahuen (*A* and *C*) and Y (*B* and *D*) strains of *T. cruzi*. After one 1 h, the mice were inoculated in the sensitization site with 10^3 trypomastigotes of the Tulahuen (*Tula*) strain. Blood was collected from tail vessels, and bloodstream trypomastigotes were counted in a hemocytometer. Control represents mice primed with vehicle medium.

sialidase gene (12). Recombinant *trans*-sialidase was purified using the same type of immunoaffinity adsorbent and eluant that is used for purification of the endogenous enzyme (9). Specifically, bacterial lysates containing recombinant *trans*-sialidase were adsorbed to insolubilized mAb TCN-2, which reacts with the COOH-terminal tandem repeat of the enzyme (29). Recombinant enzyme bound to the column was desorbed with synthetic peptide TR (DSSAHGTPSTPA), a TCN-2 hapten (29). Specific *trans*-sialidase activity of the recombinant bacterial proteins was 12–42% that of the endogenous trypomastigote enzyme (Fig. 2 *A*). By SDS-PAGE, recombinant *trans*-sialidase migrated as bands of ~200 kD as determined by staining with silver or pro-blue (Fig. 2 *B*). Recombinant *trans*-sialidases such as 17D, at 30 ng per mouse, significantly increased parasitemia and reduced survival of the Chagasic animals, similar to the effect of the trypomastigote enzyme (Fig. 2, *C* and *D*).

Specificity of the Virulence-enhancing Activity of the trans-Sialidase. Using a protocol similar to that described in Figs. 1 and 2, footpads of BALB/c mice were primed with viral and bacterial sialidases followed by an inoculum of *T. cruzi* trypomastigotes 1 h later. The viral enzyme was from Newcastle disease virus (NCV)[1] because this enzyme, like the *T. cruzi trans*-sialidase, hydrolyzes sialic acid–linked α2,3 but not α2,6 (30). The effect of the NCV sialidase was the opposite of that produced by the *trans*-sialidase. Thus, NCV sialidase–primed mice developed somewhat lower parasitemia (Fig. 3 *A*) and died slightly later (Fig. 3 *B*) than control mice, which were treated in the same way as the experimental mice, except that priming of the tissues was with vehicle medium without sialidase. Similarly, sialidase from the bacterium *V. cholerae*, which hydrolyzes sialic acid–linked α2,3 and α2,6, slightly reduced parasitemia and survival of the infected mice (data not shown), in agreement with results using culture cells (17, 31). These results suggest that the virulence-enhancing effect of the *trans*-sialidase was restricted to the *T. cruzi* enzyme.

The specificity of the *trans*-sialidase action was further demonstrated with penetrin, which is a *T. cruzi* heparin-binding protein that promotes trypomastigote adhesion to and invasion of mammalian cells in vitro (25). Unlike the *trans*-sialidase, priming of BALB/c mice footpads with penetrin, isolated either from trypomastigotes or from engineered bacteria, did not significantly alter the course of *T. cruzi* infection (Table 1)

Because LPS is a potent stimulator of immune responses, in particular of B lymphocytes (32), a trivial explanation for the virulence-enhancing effect of the *trans*-sialidase might be attributed to LPS contaminating the *trans*-sialidase preparations. We purified the *trans*-sialidase using pyrogen-free water and reagents, such that enzyme preparations had undetectable levels of LPS (<100 pg LPS/μg *trans*-sialidase) as assessed by the *Limulus* amebocyte assay. Furthermore, priming footpads of BALB/c mice with purified bacterial LPS for 1 h did not alter parasitemia, contrary to the effect of the *trans*-sialidase (Table 1).

Kinetics of the Virulence-promoting Activity of the trans-Sialidase. Sensitization of mouse soft tissues with the *trans*-sialidase (20 ng per mouse) for various times followed by inoculation of trypomastigotes into the priming site revealed that the enzyme enhanced virulence optimally after 1 h of sensitization (Fig. 4, *A* and *B*). Priming the mice with the *trans*-sialidase for as long as 24 h still produced a noticeable effect, as did injection of the enzyme together with the parasites (Fig. 4, *A* and *B*). Interestingly, injection of the *trans*-sialidase ≥1 h after the *T. cruzi* inoculum had no noticeable effect on the course of infection (Fig. 4, *A* and *B*).

The trans-Sialidase Enhanced Infection Whether It Sensitized the T. cruzi Inoculation Site or a Distinct Site. We considered the possibility that *trans*-sialidase priming enhanced parasitemia by promoting migration of trypomastigotes inoculated into the soft tissues of the mouse, which would have greater access to neighboring host cells and blood vessels. Thus, if this were the case, we should find increased parasitemia of the inoculated parasites before their invasion and replication inside host

[1] *Abbreviations used in this paper:* NCV, Newcastle disease virus; SCID, severe combined immunodeficiency.

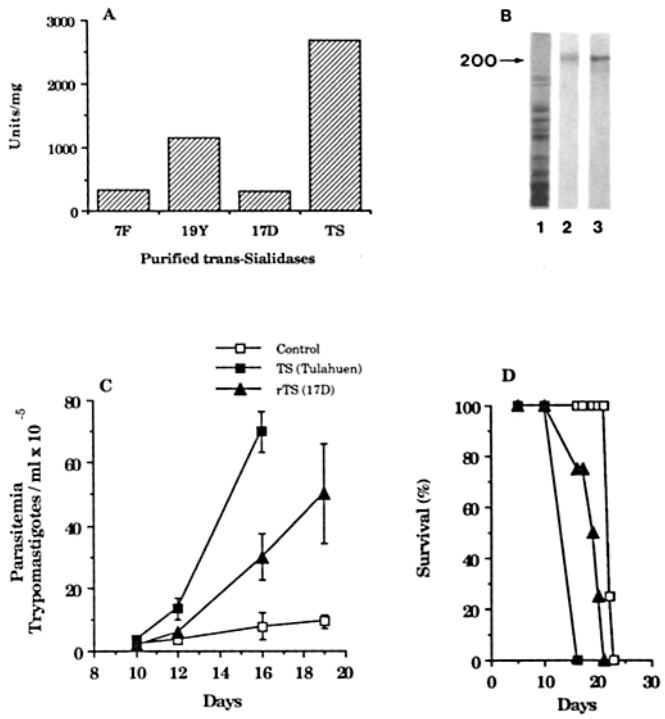

Figure 2. Characteristics of recombinant *trans*-sialidase. (*A*) Specific *trans*-sialidase activity of purified recombinant (*7F, 19Y, 17D*) and endogenous (*TS, Tulahuen*) *trans*-sialidases. (*B*) Pro-blue staining of SDS–polyacrylamide gel (7.5%) run with lysate of *E. coli* transformed with *trans*-sialidase gene (clone 7F; lane *1*) and recombinant *trans*-sialidase 7F purified on mAb TCN-2 affinity column (lane *2*). Lane *3* represents immunoblot of purified 7F probed with the *trans*-sialidase mAb TCN-2. (*C*) Parasitemia and (*D*) Survival of mice primed in the footpads for 1 h with 25 ng of endogenous (*TS, Tulahuen*) and recombinant (*17D*) *trans*-sialidases. Similar results were obtained with 7F and 19Y recombinant *trans*-sialidases.

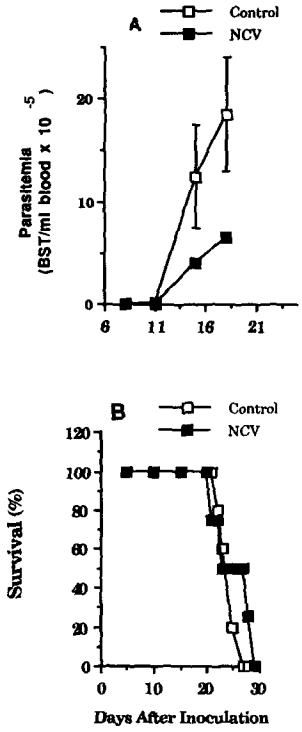

Figure 3. Effect of NCV sialidase in the course of *T. cruzi* infection in BALB/c mice. Protocol similar to that of Fig. 1, except priming of the soft tissues (footpad) was with NCV sialidase (20 U ~10 μg protein)

cells at the inoculation site. However, no such increase in early parasitemia was detected when bloodstream trypomastigotes were quantitated by various criteria, including light microscopy and PCR using a highly sensitive probe (28) (data not shown). Parasitemia augmented by *trans*-sialidase priming was observed only ~2 wk after inoculation, when the infection was presumably amplified by several cycles of trypomastigote invasion of cells in peripheral tissues.

On the other hand, exacerbation of infection was observed regardless of whether the parasites were inoculated into the site primed with the *trans*-sialidase (same footpad) or into a site distinct (different footpads as shown in Fig. 5 or footpad and intraperitoneal cavity, data not shown) from the priming site. The result shown in Fig. 5 suggests that alteration of host responses to *T. cruzi* infection is one possible mechanism for the virulence-enhancing activity of the *trans*-sialidase.

The trans-Sialidase Did Not Enhance Virulence in Severe Combined Immunodeficiency (SCID) Mice. SCID mice are devoid of functional T and B lymphocytes while having functional macrophages and NK cells (33). To determine whether the virulence-enhancing activity of the *trans*-sialidase was dependent on functional lymphocytes, we followed the course of infection of SCID mice that had been sensitized with the *trans*-sialidase (25 ng per mouse, 1-h priming). Parasitemia in the *trans*-sialidase–sensitized SCID mice was not significantly different from control mice (Fig. 6), unlike the findings with immune competent mice (Fig. 1). Furthermore, the mortality curve for the *trans*-sialidase–primed mice was the same as for the control mice (data not shown). These results suggest that the virulence-promoting activity of the *trans*-sialidase requires functional lymphocytes of the host.

Table 1. *Parasitemia of T. cruzi-infected BALB/c Mice Sensitized with a Single Dose of trans-Sialidase, Penetrin, and LPS*

	Trypomastigotes/ml blood × 10^5 at day	
Mice sensitized with	11	15
PBS (control)	10 ± 4	62 ± 20
TS (Silvio X 10/4)	18 ± 6	328 ± 60
Penetrin	16 ± 3	52 ± 12
Bacterial LPS	11 ± 1	67 ± 21

Groups of three BALB/c mice were primed in the two footpads with 25 μl of PBS (control), purified *trans*-sialidase (*TS*) of Silvio X-10/4 (1 μg/ml), recombinant penetrin (100 μg/ml), and bacterial LPS (10 μg/ml). After 1 h, 25 μl of tissue culture trypomastigotes (Tulahuen strain, 2 × 10^5/ml) were inoculated in the priming site. Mice were bled in their tail and bloodstream trypomastigotes counted in a hemocytometer on the indicated days. Lower concentrations of penetrin (10 μg/ml) and LPS (1 μg/ml) gave similar results to those shown above.

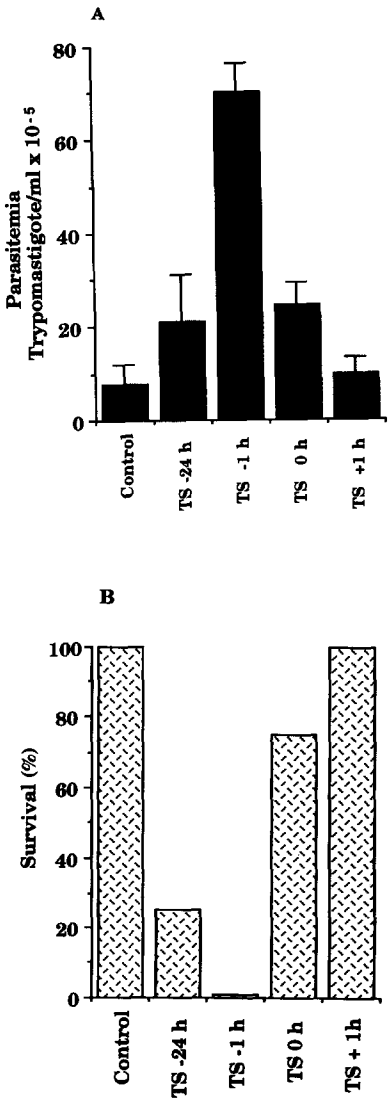

Figure 4. Kinetics of the *trans*- sialidase-induced enhancement of virulence. BALB/c mice were primed with the endogenous (*Tulahuen*) *trans*-sialidase (30 ng/mouse) for the indicated times and inoculated with 2 × 10^3 trypomastigotes. Control represents mice primed for 1 h with vehicle medium. Parasitemia (*A*) and survival (*B*) are displayed for the 16th d after inoculation.

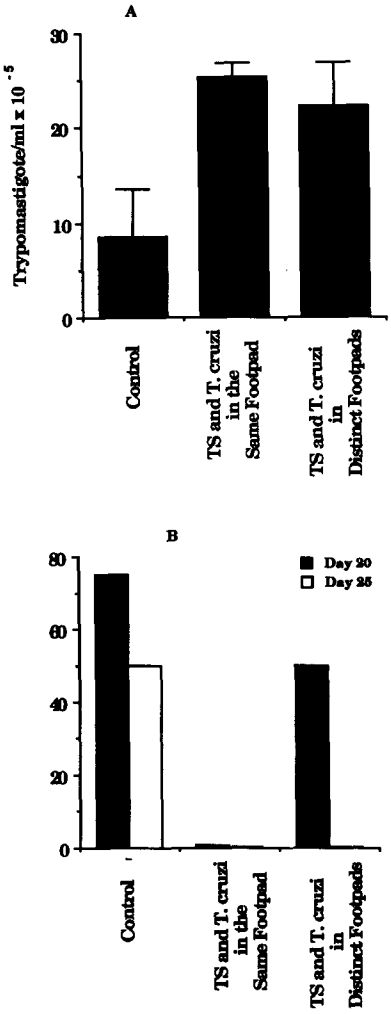

Figure 5. *Trans*-sialidase enhances virulence where priming and inoculation sites are physically apart. BALB/c mice were primed with endogenous *trans*-sialidase and inoculated with trypomastigotes 1 h later in the priming site (same footpad) or in a distinct site (the other footpad). Parasitemia at day 20 (*A*) and survival (%) at days 20 and 25 (*B*) are displayed.

trans-Sialidase Priming Did Not Boost Antibody Response to T. cruzi. One explanation for the enhancement of *T. cruzi* growth in BALB/c mice is that enzyme priming would lower humoral and cell-mediated immunity to the parasite during the course of infection. To test if antibody response to *T. cruzi* was altered by *trans*-sialidase priming, we measured by ELISA the antibody titers against *T. cruzi* in the sera of primed and nonprimed control BALB/c mice infected with Tulahuen trypomastigotes. As shown in Fig. 7, titers of antibodies against *T. cruzi* trypomastigotes were not affected by *trans*-sialidase priming, in contrast to the parasitemia in primed and control animals. Thus, at least in BALB/c mice, *trans*-sialidase–induced enhancement of virulence cannot be attributed to reduced antibody response to *T. cruzi*.

Effect of trans-Sialidase Antibodies on the Course of T. cruzi Infection in BALB/c Mice. Earlier studies showed that polyclonal and monoclonal *trans*-sialidase antibodies enhanced *T. cruzi* infection of fibroblasts and smooth muscle cells in vitro (14, 20). One of the enhancing mAbs, TCN-2, was specific for the tandem repeat in the COOH terminus of the *trans*-sialidase (29). To test whether these antibodies were also active in vivo, BALB/c mice were inoculated with *T. cruzi* in the presence of 24.5 μg of protein G–purified mAb TCN-2. Remarkably, administration of TCN-2 greatly enhanced parasitemia and mortality compared with control mice that received isotype-matched IgG instead of TCN-2 (Fig. 8, *A* and *B*). The monovalent Fab fragment of TCN-2 (18.8 μg per mouse) also increased *T. cruzi* virulence (Fig. 8 *A*), suggesting that the effect of TCN-2 was not caused by Fc-mediated endocytosis of the parasite by macrophages and other cells.

We also studied the effect of antibodies against the Cys-

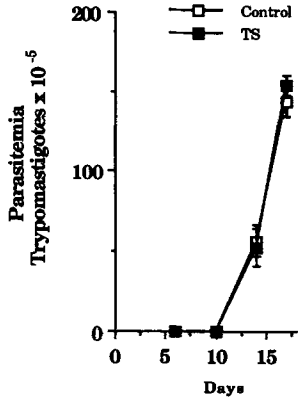

Figure 6. Effect of *trans*-sialidase priming in SCID mice infected with *T. cruzi*. Protocol similar to that of Fig. 1 except the parasite inoculum was 200, instead of 1,000 trypomastigotes.

rich part of the *trans*-sialidase in vivo. Most experiments were done with antibodies against peptide C (^{381}RLPKRMGGSYRC^{392}, numbering from the recombinant *trans*-sialidase 7F, ref. 12). Two types of peptide C antibodies were used: antibodies raised in mice by immunization with the synthetic peptide in the presence of Freunds' adjuvant, and antibodies from mouse or human Chagasic sera. Affinity purification of the antibodies was by adsorption to peptide C coupled to Sepharose beads and specific elution with soluble synthetic peptide C. The affinity-purified peptide C antibodies inhibited *trans*-sialidase activity and depleted enzyme from trypomastigote lysates after adsorption to protein G–Sepharose (Ming, M., and M. E. A. Pereira, manuscript in preparation).

All types of peptide C antibodies aborted the course of *T. cruzi* infection in BALB/c mice, although the peptide C antibodies isolated from human Chagasic sera were the most potent. A concentration as low as 9.6 μg of the human antibody per mouse dramatically reduced parasitemia to barely detectable levels (Fig. 8 *B*), and it completely protected mice from the lethal inoculum of *T. cruzi* (Fig. 8 *D*). In addition, peptide C antibodies were also very active in abrogating *T. cruzi* infection in vitro (Ming, M., and M. E. A. Pereira, manuscript in preparation). Interestingly, vaccination with peptide C reversed the virulence of *T. cruzi* for BALB/c mice (Ming, M., and M. E. A. Pereira, manuscript in preparation). The results with the *trans*-sialidase antibodies further support the inference that the enzyme is a virulence factor in Chagas' disease.

Discussion

We show here that sensitization of mice with *T. cruzi trans*-sialidase followed by an inoculum of Tulahuen trypomastigotes in the priming site or in a distinct site significantly enhanced parasitemia and mortality of the infected animals. Both endogenous and recombinant enzyme were effective in augmenting virulence. The enhancement was specific because viral and bacterial sialidases were inactive, as was penetrin (Table 1), a heparin-binding protein of *T. cruzi* that, as the *trans*-sialidase, promotes trypomastigote attachment to host cells (25). In another system, the *V. cholerae* sialidase was reported to promote intracellular killing, rather than survival, of microbes in vivo (34).

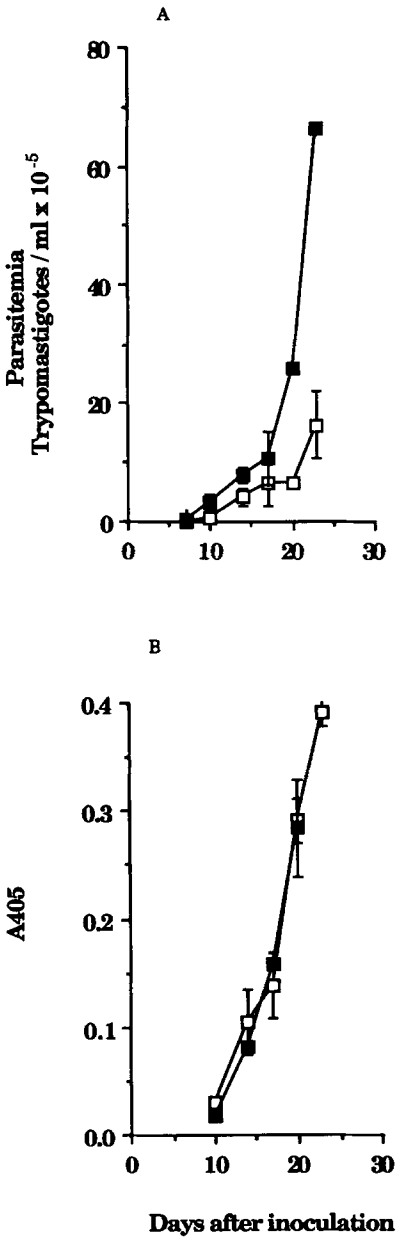

Figure 7. *trans*-sialidase priming did not alter titers of antibodies to *T. cruzi* in Chagasic mice. BALB/c mice were primed with vehicle medium (control) and *trans*-sialidase as in Fig. 1. At the indicated days, parasitemia was determined by light microscopy and antibody elicited to trypomastigotes estimated by ELISA. —□—, control; —■—, TS.

The *trans*-sialidase is anchored to the plasma membrane of trypomastigotes by a glycophosphoinositol anchor (11), and consequent to the action of endogenous and environmental phospholipases, the enzyme is readily shed in the environment inhabited by the trypanosomes, including tissues of mice (3) and humans (16) infected with *T. cruzi*. Therefore, soluble enzyme released in vivo during infection of mammals might exert an effect analogous to the one reported here. Quantitative considerations further suggest that the enhancement of virulence in the mouse model is of physiological significance,

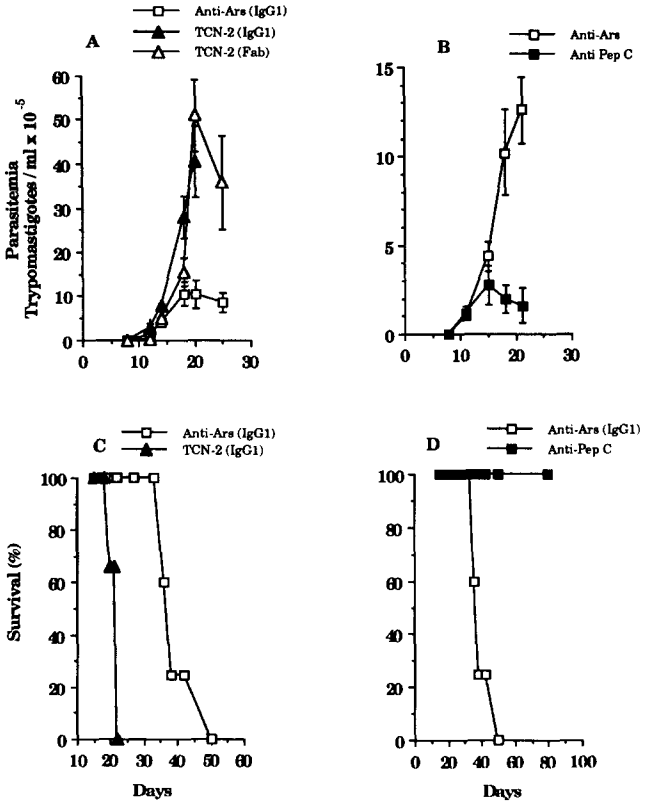

Figure 8. Effect of passive administration of *trans*-sialidase antibodies on the course of *T. cruzi* infection. Tulahuen trypomastigotes were incubated with the indicated antibodies for 1 h at room temperature and inoculated subcutaneously into the footpad of BALB/c mice. Anti-Ars, anti-*p*-nitrophenylarsonate.

as it was achieved with very small amounts of enzyme, ~1–5 μg/kg or low picomole range per kilogram. Such small amounts of *trans*-sialidase could be shed by *T. cruzi* in the acute phase of Chagas' disease, where parasites are abundant in peripheral tissues and in the circulation (1, 2).

One explanation for enhanced parasite thriving in the primed mice relates to a direct interference of the *trans*-sialidase in *T. cruzi*–host cell associations, similar to the enzyme effect in vitro (17). However, direct interference is an unlikely explanation because virulence was enhanced when enzyme priming and parasite inoculation sites were physically distinct from one another, such as in different footpads or a footpad and intraperitoneal cavity (Fig. 5). Kinetic data (Fig. 4) are also inconsistent with such a mechanism, for enhancement was optimum after *trans*-sialidase priming for 1–2 h and negligible when the enzyme was injected after the parasites. In addition, the amount of enzyme needed to induce virulence (~20–30 ng per mouse) seemed to be too small to effectively diffuse through the tissues and directly interfere with *T. cruzi* binding to host cells. Instead, the results are consistent with the *trans*-sialidase indirectly stimulating parasite growth in vivo. This would occur if the *trans*-sialidase would bind to receptors on cells of the immune system and trigger a cascade type of reaction, resulting in reduced host response against the parasite.

Host cells that might indirectly promote invasion are those that participate in the inflammatory response, such as leukocytes and macrophages. These cells become activated after reaction of surface receptors with specific ligands in the extracellular environment. Many surface receptors are sialylated glycoproteins, which send signal-transducing signals not only after engagement with specific ligands, but also after reaction with the plant lectins Con A and PHA. Because the *trans*-sialidase is a sialic acid–binding protein, it could activate inflammatory cells by binding to sialic acid in the carbohydrate side chains of a given receptor.

One candidate receptor is CD45, which is the most abundant glycoprotein on leukocytes, where it comprises ~10% of the T lymphocyte surface (35, 36). It is a member of a novel class of enzymes, named protein tyrosine phosphatase, and as such, CD45 is linked to reversible protein tyrosine phosphorylation (37). Tyrosine phosphorylation is a key regulatory mechanism controlling growth and division of eukaryotic cells (38). The extracellular domain of CD45 is heavily glycosylated, bearing many N- and O-linked carbohydrates containing α2,3-linked sialic acid (35, 36), precisely the structures recognized by the *trans*-sialidase (8, 9). Interestingly, CD45 carbohydrates on T lymphocytes interact with the plant lectin PHA, inducing T cells to enter the S phase of the cell cycle (39).

It is not unreasonable to think that the *trans*-sialidase will bind to inflammatory cell receptors, since it reacts with sialic acid on the surface of erythrocytes and endothelial cells (40, 41), on soluble glycoproteins such as orosomucoid and fetuin (5), and on oligosaccharides (8, 9). However, only experiments will tell whether *trans*-sialidase binding to inflammatory cells will lead to their activation or deactivation. It is of interest to note that purified *trans*-sialidase stimulates DNA synthesis (i.e., [^{3}H]thymidine incorporation) in cultures of nylon wool–purified naïve lymphocytes of mouse and human origin (Ming, M., and M. E. A. Pereira, unpublished observations). Consistent with this hypothesis, *trans*-sialidase sensitization followed by *T. cruzi* infection of SCID mice that lack functional B and T lymphocytes (33) failed to enhance parasitemia and mortality (Fig. 6). These results are in agreement with the hypothesis that the *trans*-sialidase enhanced virulence in the animal model by altering immune responses critical to combat invasion of *T. cruzi* and perhaps of other parasites. However, *trans*-sialidase priming did not suppress or enhance antibody response to the trypanosomes in the Chagasic mice (Fig. 7), suggesting that virulence enhancement was not caused by an alteration in the amount of antibody produced against the parasite. We do not know yet if the priming altered cell-mediated immunity to *T. cruzi*. In another system, the *V. cholerae* sialidase was reported to enhance antibody response in vivo to various antigens (42).

It is implicit in this hypothesis that the virulence-enhancing effect is mediated by the sialic acid–binding site of the *trans*-sialidase. However, because the *trans*-sialidase is a multidomain protein (12), it is not inconceivable that the enzyme

exerts biological activities through a site separate from the catalytic region. One such site is the fibronectin type III domain, which is present not only in the *trans*-sialidase, but also in other *trans*-sialidase family members (4). Fibronectin type III domains of fibronectin and other adhesion proteins participate in various types of protein–protein interactions (43), and by analogy, the *trans*-sialidase unit might have a similar role.

The *trans*-sialidase may behave like other microbial adhesive proteins that, on one hand, directly participate in host–parasite interactions, and on the other hand, indirectly contribute to the infective process by altering physiological parameters of the mammalian host. One example of such proteins is the coat gp120 of HIV, which recognizes CD4 receptor molecules on T lymphocytes during viral adherence and entry (44). In addition, gp120 triggers important biological activities thought to be related to the pathology of HIV infection. Soluble gp120 reacts with monocytes, inducing the release of cytokines and arachidonic acid metabolites, which may affect the function of immunocompetent cells (45), and it kills cortical neurons at low picomolar concentrations by stimulation of nitric oxide and superoxide anions, an activity that could be related to AIDS dementia (46). Another example is HIV Tat protein, which stimulates viral gene expression by binding to specific promoters (47). In addition, Tat has profound effects on mammalian cells such as induction of proliferation and TGF-β expression in chondrocytes (48).

The effect of passive administration of *trans*-sialidase antibodies in vivo are of interest. The enhancement of infection produced by mAb TCN-2 is consistent with early findings in vitro, which showed this antibody, as well as rabbit polyclonal antibodies, to increase *T. cruzi* binding and invasion of mammalian cells in culture (14, 20). This enhancement may not result from facilitation of Fc-mediated endocytosis of the parasite, because the monovalent Fab fragment of TCN-2 was as active as the bivalent IgG (Fig. 7 *A*). The remarkable effect of TCN-2 in vivo may perhaps be related to the fact that the antibody was obtained from the spleen of a mouse chronically infected with *T. cruzi* and not artificially immunized with the enzyme (20). Consequently, tandem repeat antibodies could have a similar effect in Chagasic individuals. Most patients with acute Chagas' disease develop antibodies against the *trans*-sialidase tandem repeat (49), and it is not inconceivable that tandem repeat antibodies found in natural infections could have a positive effect in parasite growth in the mammalian host.

But not all antibodies enhanced infection. Antibodies against an amino acid sequence (peptide C) present in the Cys-rich region of the *trans*-sialidase was remarkably potent in reducing infection when administered subcutaneously with the parasites. Most active were those peptide C antibodies isolated from a pool of sera of patients with indeterminate and chronic Chagas' disease. Thus, antibodies against various domains of the *trans*-sialidase elicited in vivo during natural infections may influence parasitism in opposite ways. This would not be unique, since antibodies against the lectin of *Entamoeba histolytica* had an effect similar to the *trans*-sialidase antibodies in that they either enhanced or blocked lectin-mediated cytolysis of cultured epithelial cells (50).

In conclusion, the results presented here show that priming soft tissues with *trans*-sialidase enhances *T. cruzi* infection in mice. We hypothesize that the enhancement results from the activation/deactivation of host inflammatory cell responses. We propose that when *T. cruzi* infects a mammalian host, the trypomastigote-bound *trans*-sialidase binds to sialyl residues on the surface of fibroblasts and other host cells as a prelude to attachment and penetration. At the same time, *trans*-sialidase released by trypomastigotes into the environment binds to receptors (most likely α2,3-sialyl epitopes) and modifies host functions such as those of inflammatory cells, ultimately resulting in enhanced invasion. This model is consistent with the augmentation and diminution of infection in vivo by *trans*-sialidase antibodies.

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