Invasive Phenotype of *Trypanosoma cruzi* Restricted to a Population Expressing *trans*-Sialidase

MIERCIO E. A. PEREIRA,* KEMING ZHANG, YUHONG GONG,
ENRIQUE M. HERRERA, AND MAO MING

*Tropical Diseases Research Unit, Departments of Medicine and Pathology,
Tufts-New England Medical Center, Boston, Massachusetts 02111*

Received 18 March 1996/Returned for modification 10 May 1996/Accepted 1 July 1996

Trypanosoma cruzi expresses a developmentally regulated *trans*-sialidase implicated in the pathogenesis of Chagas' disease. On inhabitation of the extracellular milieu of cultured cells by infective trypomastigotes, the enzyme is restricted to a small (20 to 30%) population of parasites. The biological significance of *trans*-sialidase expression on this subset, termed TS⁺, and not on the majority (70 to 80%) of morphologically similar trypanosomes, named TS⁻, is unknown. To determine the roles of the TS⁺ and TS⁻ subsets in *T. cruzi* invasion, we prepared pure populations of TS⁻ and TS⁺ trypanosomes using magnetic beads coated with a monoclonal antibody specific for the tandem repeat unit of the *trans*-sialidase C terminus. After removal of nonadherent TS⁻ trypomastigotes, the TS⁺ trypomastigotes were isolated from the beads by specific elution with a synthetic peptide epitope of the *trans*-sialidase monoclonal antibody. Confirmation of TS⁺ and TS⁻ phenotypes was obtained by immunofluorescence, immunoblotting, and sialidase or sialyl transferase activity measurements. The TS⁺ trypanosomes were highly invasive, as they attached to, penetrated, and thrived in cultured mammalian cells much more efficiently than did unfractionated parasites. The critical role of the *trans*-sialidase in invasion was underscored by the observation that infection was neutralized by human antibodies to *trans*-sialidase. What's more, the TS⁻ parasites, in sharp contrast to their TS⁺ counterparts, were extremely inefficient in invading epithelial cells and fibroblasts. Further, introduction of small amounts of exogenous *trans*-sialidase into suspensions of nonpenetrating TS⁻ parasites converted them to a highly invasive phenotype indistinguishable from that of the TS⁺ population. Rescue of the invasive phenotype was specific for the *T. cruzi* enzyme, for it didn't happen with bacterial and viral sialidases. The *in vitro* results were confirmed in the murine model of Chagas' disease, as TS⁻ trypomastigotes were relatively avirulent while TS⁺ trypomastigotes were more virulent than unfractionated parasites.

A given population of mammalian cells usually presents a uniform appearance by light and electron microscopy. Despite their homogeneous morphology, the cells may comprise many functional subpopulations, usually identified and distinguished from one another by the differential expression of cell surface protein markers. The functional heterogeneity of morphologically similar cells is particularly striking in lymphocytes, where B and T cells are identified and separated from each other by antibodies to the B- and T-cell antigen receptors (11). T lymphocytes can be further subdivided by the selective expression of CD4 and CD8 proteins, to give helper T cells and cytotoxic T cells, respectively (11). It would be difficult to envision the great progress in understanding the physiology and malfunction of the immune system in the last two decades without methodologies to identify and separate lymphocytes bearing unique phenotypic markers (11).

By analogy with mammalian cells, it may very well be that morphologically defined forms of protozoan parasites consist of discrete populations expressing distinguishing plasma membrane proteins. If so, each population could play a unique role in host-parasite interactions. Identifying and understanding how parasite populations interact with the host and with one another might be important to devise strategies to combat parasitic diseases. The hypothesis that a given parasite stage comprises subsets with unique effector functions can be tested

with *Trypanosoma cruzi*, the etiological agent of Chagas' disease.

T. cruzi infects mammalian cells through two developmental forms, amastigote and trypomastigote. Amastigotes replicate and differentiate into nondividing trypomastigotes inside the host cell cytoplasm. Concomitant with this differentiation is the expression of *trans*-sialidase, an enzyme that recognizes sialic acid in α 2,3 linkage and that has both sialidase (neuraminidase) (2) and sialyltransferase (17, 26) activities. Trypomastigotes, while in the cytosol or exiting infected cells, all bear the enzyme (23). However, in trypomastigotes living in the extracellular milieu, *trans*-sialidase is expressed in a small (20 to 30%) subset, named the TS⁺ population, and not in the majority (70 to 80%) of parasites, termed TS⁻ (4, 21).

Trypomastigotes bind to and enter many types of mammalian cells through receptor-mediated endocytosis (19). They spread infection by invading neighboring cells or distant cells through the circulation. Under natural conditions, they transmit infection to other hosts through reduviid insects (2). Trypomastigote recognition of and entry into host cells depend on the expression of several molecules, including *trans*-sialidase (19). The enzyme, presumably when located on the surface of trypomastigotes, mediates adhesion by reacting with α 2,3-sialyl determinants on the outer membrane of host cells (14). Human antibodies reactive with the *trans*-sialidase neutralize infection *in vivo* (5). In addition, soluble enzyme may promote parasitism *in vivo* through a mechanism independent of direct *T. cruzi*-host cell binding (5).

Thus, trypomastigotes invade cells while expressing *trans*-sialidase in the small TS⁺ population. We sought to determine

* Corresponding author. Mailing address: Tropical Diseases Research Unit, Departments of Medicine and Pathology, Tufts-New England Medical Center, 750 Washington St., Box 41, Boston, MA 02111. Fax: (617) 636-5292.

whether a parasite population, defined on the basis of a phenotypic marker (*trans*-sialidase) for which a monoclonal antibody (MAb) is available, might be the actual perpetrator of invasion and, if so, whether the phenotypic marker was functional in trypanosome-host cell interactions. To ascertain the function of cell or parasite populations requires first their physical separation from one another. However, in contrast to the situation with sorting of lymphocytes, methodologies to isolate populations of *T. cruzi* and of other parasites on the basis of phenotypic markers are not readily available.

Here we report the rapid separation of TS⁺ from TS⁻ trypomastigotes by exposing the parasites to magnetic beads coated with a MAb (TCN-2) that recognizes the *trans*-sialidase C-terminal domain. The TS⁻ trypanosomes, purified by negative selection after subjection of the beads to a magnetic field, were extremely poor invaders in vitro and in vivo. In contrast, the TS⁺ trypomastigotes, isolated from the beads by specific elution with a TCN-2 epitope, were highly invasive for cells in culture and virulent for experimental animals. These results establish that trypomastigotes carrying the phenotypic marker *trans*-sialidase are the de facto invaders of cells. What's more, TS⁻ trypomastigotes acquired an invasive phenotype by reconstitution with small amounts of exogenous purified *trans*-sialidase, further underscoring the critical role of the enzyme in *T. cruzi* invasion.

MATERIALS AND METHODS

Parasites and cell lines. All invasion and attachment assays in vitro were performed with the *T. cruzi* clone Silvio X-10/4, and all experiments in vivo were performed with the clone Tulahuen C2. These clones were described previously, as was their propagation in Vero cells (4, 21, 23). The Mv1Lu mink lung cell line and Vero cells were from the American Type Culture Collection.

Antibodies. MAb TCN-2 (immunoglobulin G1 [IgG1]) was isolated from a C3H mouse chronically infected with a clone of MV-13 that expresses high-level *trans*-sialidase activity (21). The antibody was purified from hybridoma supernatants on protein G-Sepharose columns (Pharmacia) equilibrated with phosphate-buffered saline (PBS), pH 7.5; bound antibodies were eluted with 0.1 M glycine-HCl buffer, pH 2.5, and immediately dialyzed against PBS. The isotype-matched control antibody was a hybridoma against *p*-azophenylarsenate (21), which was also purified on protein G-Sepharose as described above.

Human antibodies to peptide C (³⁸⁴RLPKRMGGSYRC³⁹² [20]) were purified as described elsewhere (5) from Chagasic sera, which consisted of a pool of sera from individuals with indeterminate and chronic Chagas' disease from Bahia, Brazil, an area in which Chagas' disease is endemic. Purification of peptide C antibodies from Chagasic sera was done by coupling peptide C to Sepharose 4B activated with cyanogen bromide (~3 to 5 mg of peptide per ml of settled beads). IgG from Chagasic sera bound to the peptide C-Sepharose column was specifically eluted by addition of soluble synthetic peptide (2 mg/ml) to the equilibrating PBS buffer. Peptide C was separated by the antibody by extensive dialysis. IgG from non-Chagasic individuals was used as a control for the peptide C antibodies.

Isolation of TS⁺ and TS⁻ trypomastigotes on magnetic beads. Dynabeads M-450 with covalently bound, affinity-purified sheep Ig against mouse IgG (Dyna) were saturated with *trans*-sialidase antibodies by incubating the beads (4 × 10⁸ in 1 ml) with culture supernatants of hybridoma TCN-2 (13 ml) for ~14 h at 4°C in a 360° rotary shaker. The bead suspension was subjected to a magnetic field (MPC-1; Dynal) for 1 to 2 min, the supernatant was pipetted out, and the beads were reincubated with fresh TCN-2 supernatant for another 2 to 3 h as described above. About 0.5 to 3 µg of MAb TCN-2 bound to 1.0 ml of beads. Beads were washed with sterile 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered (pH 7.4) RPMI containing 1% bovine serum albumin (RPMI-BSA) and separated with the MPC magnet. Washing was repeated three times. The TCN-2-coated beads were then mixed with 1 × 10⁸ to 3 × 10⁸ trypomastigotes in RPMI-BSA (total volume, 8.0 ml) and incubated for 1 h at 4°C in a rotary shaker. Beads containing adherent parasites were separated from nonadherent parasites with the magnet. The negatively selected parasites were reapplied to fresh TCN-2-coated beads for 1 h and isolated as described above. The positively selected parasites were washed twice with RPMI-BSA and incubated in the same medium containing synthetic peptide TR (DSSAHGTP STPA; 10 mg/ml) for 1 h at 4°C in a 360° rotary shaker to elute trypanosomes adherent to the TCN-2-coated beads. Synthetic peptide TR is an epitope of MAb TCN-2 (22). In some experiments, the beads were incubated with a synthetic peptide modeled from *trans*-sialidase sequence that does not react with TCN-2 (peptide B; ¹⁵²TNKRKQVFSKIF¹⁶³) (20) to test the specificity of the elution by peptide TR (see Fig. 1). After separation of the beads from the eluted parasites

with a magnet, the positively and negatively selected trypanosomes were washed three times by centrifugation (1,000 × *g*, 10 min, 4°C) in RPMI-BSA. The parasite populations, after being counted in a hemocytometer, were immediately used in the various assays described below or frozen at -70°C.

Adhesion, penetration, and infection assays. Adhesion of trypomastigotes to monolayers of glutaraldehyde-fixed Vero or Mv1Lu cells was performed as previously described (14-16). Briefly, trypomastigotes (10⁵ per well containing 0.1 ml of RPMI-BSA) were incubated at 37°C with glutaraldehyde-fixed monolayers in 16-well Lab-Tek chamber slides. After 1 h, swimming and loosely bound parasites were removed by dipping the slides 10 times in PBS. Adherent parasites were fixed for 10 min with 4% paraformaldehyde in PBS containing 7% sucrose. After a rinse with PBS, the slides were stained with Diff-Quik and examined under a light microscope to assess *T. cruzi* adherence. A minimum of 300 host cells were counted for each point.

For penetration (15, 16), monolayers of the various cell types, equilibrated in RPMI-BSA medium, were incubated with trypomastigotes (6 × 10⁶/ml) for 1 or 2 h at 37°C, washed in cold HEPES-buffered (pH 7.2) RPMI, fixed in 2% paraformaldehyde in 100 mM lysine for 20 min, and blocked with 50 mM ethanolamine. The monolayers were fixed one more time in 1% glutaraldehyde for 1 h at room temperature and blocked with 1% BSA overnight at 4°C. They were then reacted for 30 min with IgG (20 µg/ml) purified from human Chagasic sera on protein G-Sepharose (5) and washed several times with PBS, after which the bound IgG was detected with a fluorescein isothiocyanate (FITC)-labeled second antibody (Calbiochem). After a wash with PBS, the DNA of the host cells and the parasites was stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma) at 1 µg/ml and observed under a Nikon epifluorescence microscope.

Infection of cultured cells was carried out as described previously (14-16). Subconfluent monolayers were incubated for 1 to 2 h at 37°C with various concentrations of TS⁺, TS⁻, and unfractionated trypomastigotes in 0.1 ml of RPMI-BSA. The monolayers were washed with RPMI to remove swimming parasites and further incubated for 2 to 3 days at 37°C in RPMI-1640 containing 5% fetal calf serum. Diff-Quik-stained preparations were examined under a microscope to estimate the ratio of infected cells (i.e., cells containing more than one intracellular amastigote) to noninfected cells.

Assay and purification of *trans*-sialidase. For the *trans*-sialidase assay (27), enzyme either purified or in crude preparations in conditioned supernatants and in parasite lysates was added in a 10-µl volume to 40 µl of 50 mM PBS, pH 7.2, containing 0.5 mmol of 2,3-sialyllactose (Sigma), 0.25 mmol of [¹⁴C]-N-acetyllactosamine (4 × 10⁵ dpm), 50 µg of BSA, 0.5% Triton X-100, and 0.02% NaN₃. 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 of Q-Sepharose equilibrated with water. Sialylated product was eluted with 1 M NaCl and quantitated by scintillation counting. For the purification of *T. cruzi trans*-sialidase (5, 27), conditioned supernatants of Vero cell cultures infected with *T. cruzi* Silvio X-10/4, 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 ultrafiltration on a Centri-con-10 concentrator (Amicon Corp.).

Other procedures. (i) Immunoblotting. Immunoblotting was performed with nitrocellulose containing parasite lysates (~10 µg per lane) run on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Blots were probed with MAb TCN-2 and developed with alkaline phosphatase substrates (21); quantitation of the immunoblot product was done by using a scanning densitometer (Bio-Rad).

(ii) Immunofluorescence. Immunofluorescence assays were performed with parasites fixed in 4% paraformaldehyde, probed with culture supernatants of hybridoma TCN-2 (21) or with mouse antibodies to penetrin (9, 16) followed by an FITC-labeled second antibody (Calbiochem).

(iii) TGF-β-like activity. Transforming growth factor β (TGF-β)-like activity in *T. cruzi* was determined in the Mv1Lu cell line stably transfected with the plasminogen activator inhibitor 1 (PAI-1) promoter adjacent to a luciferase reporter, as described previously (15). Monolayers were infected overnight (~14 h) with trypomastigote populations and epimastigotes in 5% fetal calf serum.

(iv) Infection of mice. Infection of BALB/c mice (8 to 10 weeks old) was by intraperitoneal injection of Tulahuen C2 parasites (unfractionated, TS⁺, and TS⁻) in the range of 1,000 to 6,000 trypomastigotes per mouse, as described previously (5). Mice were bled in the tail at various times postinoculation, and bloodstream trypomastigotes were quantitated in a hemocytometer under a light microscope.

RESULTS

Isolation of TS⁺ and TS⁻ trypomastigotes. Rapid and reproducible separation of TS⁺ from TS⁻ trypomastigotes was achieved by exposing the parasites to magnetic beads coated with TCN-2. TCN-2 is a MAb specific for the tandem repeat in the C terminus of the *trans*-sialidase (22). In a typical experiment, when 2 × 10⁸ extracellular trypomastigotes were mixed with 4 × 10⁸ TCN-2-coated beads for 30 min, 1.50 × 10⁸ parasites (75%) were negatively selected by subjecting the

FIG. 1. Separation of trypomastigote populations on immunomagnetic beads. (A) Elution profile. Beads coated with MAb TCN-2 were exposed to trypomastigotes at 4°C in a 360° rotary shaker. After 1 h the beads were subjected to a magnetic field. Parasites not adherent to the beads (Unbound) were pipetted out into a sterile tube and counted. Parasites bound to the beads were eluted with TCN-2 epitope (Pep TR). An irrelevant synthetic peptide (Pep B) did not elute bound parasites. (B) Visualization of trypomastigotes adhering to the beads. After separation of the unbound population, the beads were fixed with 4% paraformaldehyde, reacted with human Chagasic serum, and developed with an FITC-labeled anti-human Ig. Magnification, $\times 230$. (C) Same as panel B but at higher magnification ($\times 1,250$). The diameter of the beads (in both panels B and C) is 4.2 μm . (D) Same as panel B except the beads were coated with an isotype-matched anti-*p*-azophenylarsonate MAb. Note the absence of trypanosomes adherent to the control beads.

beads to a magnetic field (Fig. 1A). The parasites positively selected on the beads (Fig. 1B and C) were purified by elution with synthetic peptide TR, the epitope of MAb TCN-2 (9), resulting in 0.31×10^8 parasites (15%) (Fig. 1A). Thus, the recovery of negatively selected plus positively selected trypomastigotes was $\sim 90\%$.

Specificity of the elution was demonstrated by the inability of an irrelevant synthetic peptide (modeled from the *trans*-sialidase sequence but which does not react with TCN-2) to significantly desorb adherent trypomastigotes (Fig. 1A). In addition, *T. cruzi* did not adhere to beads coated with an irrelevant MAb having the same isotype as TCN-2 (Fig. 1D).

The trypomastigote subset isolated by negative selection had a TS⁻ phenotype, as defined by the weak or negative reaction of these parasites with *trans*-sialidase MAbs in immunofluorescence (Fig. 2A and E) and immunoblotting (Fig. 2B and G) experiments. The weak bands visible in Fig. 2G were detected only after overexposure (8 h) of the blot to alkaline phosphatase substrates. In addition to having these immunological properties, the negatively selected trypanosome subset had a very low or barely detectable level of *trans*-sialidase activity (Fig. 2C). However, the parasites had detectably significant levels of *trans*-sialidase mRNA, albeit less than the amount in the unfractionated parasites (Fig. 3).

In contrast, trypomastigotes isolated by positive selection

had the TS⁺ phenotype, because they all ($\sim 99\%$) reacted with *trans*-sialidase antibodies in immunofluorescence experiments (Fig. 2A and F). In addition, lysates of the positively selected trypanosomes reacted with *trans*-sialidase antibodies to give a product ~ 15 and 3 times the size of the equivalent product from TS⁻ and unfractionated parasites, respectively (Fig. 2B and G). What's more, the specific *trans*-sialidase activity of the positively selected population was about 20 and 3 times higher than the enzyme activities of TS⁻ and unfractionated parasites, respectively (Fig. 2C).

Thus, immunological criteria and enzymatic analysis establish that trypanosomes adherent to and eluted from the TCN-2-coated beads were selected on the basis of the *trans*-sialidase they bear, in contrast to the nonadherent trypanosomes.

TS⁺ trypomastigotes are highly invasive in cultured cells whereas TS⁻ trypomastigotes are not. *T. cruzi* trypomastigotes attach to and enter most mammalian cells, where they differentiate into amastigotes, which divide in the cytoplasm. Amastigotes transform into trypomastigotes, which exit cells ruptured by parasite overload. We sought to determine the relative efficiencies of TS⁺ and TS⁻ trypomastigotes in each of these phases of cellular invasion.

Attachment was measured by determining the ratio of parasites bound to cells made impermeable to penetration by fixation with glutaraldehyde (14–16). We found that TS⁺ try-

FIG. 2. Immunological and enzymatic characterization of trypomastigote populations. (A, D, E, and F) Indirect immunofluorescence produced by MAb TCN-2 on unfractionated (Unf) (A and D), negatively selected (TS^-) (A and E), and positively selected (TS^+) (A and F) populations from the experiment shown in Fig. 1. Proportion of trypomastigotes (Tryps) considered to be stained by TCN-2: two of seven in the Unf population (D), zero of seven in the TS^- population (E), and seven of seven in the TS^+ population (F). (B and G) Immunoblot reaction of trypomastigote lysates with MAb TCN-2. The same amount of protein (10 μ g of protein per lane) for each lysate was run on SDS-polyacrylamide gels (7.5% polyacrylamide), transferred to nitrocellulose paper, and reacted with TCN-2. The reaction shown in panel G was quantitated in a scanning densitometer, and results are plotted in panel B. Upper and lower arrows indicate bands of 200 and 97 kDa, respectively. (C) *trans*-Sialidase activity. Live parasites were added to *trans*-sialidase reaction mixtures containing 0.5% nonionic detergent (Triton X-100).

trypomastigotes were about two times more efficient in attaching to fibroblast Vero cells than unfractionated parasites, which in turn were somewhat better than TS^- trypomastigotes (Fig. 3A).

To determine whether the attached parasites could penetrate the host cells, monolayers of Vero cells were first incubated with trypomastigotes for 1 h to allow penetration, washed to remove unbound parasites, and then fixed with glutaraldehyde to render the host cell outer membrane impermeable to macromolecules (15, 16). Penetration was assessed by the differential staining of the monolayers with a permeable dye (DAPI) and an impermeable macromolecule (IgG) reactive with *T. cruzi*. Following the trend in attachment, TS^+ trypomastigotes entered the monolayer at a rate faster than that of unfractionated trypanosomes (Fig. 3B). Unexpectedly, TS^- trypomastigotes were extremely inefficient in penetrating the host cells (Fig. 3B), even though they attached to them relatively well (Fig. 3A).

The penetration described above was determined after incubation of the parasites with the host cells for 1 h (Fig. 3B) or 2 h (data not shown). To determine whether the trypomastigotes that entered the cells were able to differentiate and multiply, the monolayers were grown for 3 days at 37°C instead of 1 to 2 h and then fixed and stained with Diff-Quik to facilitate counting of cells containing >1 amastigote. The results confirmed that TS^+ trypomastigotes are highly invasive

under conditions in which TS^- trypomastigotes are not (Fig. 3C).

Trypomastigotes released from cells infected with the TS^+ population had a TS^+/TS^- population ratio (23% TS^+ and 83% TS^-) indistinguishable from that of trypomastigotes derived from cells infected with unfractionated parasites. This result suggests that a TS^+ parasite reversibly transforms into a TS^- parasite after a cycle of invasion inside the cell.

Human *trans*-sialidase antibodies block invasion of TS^+ trypomastigotes. We recently showed that antibodies against peptide C present in the Cys-rich domain of *trans*-sialidase (clone 7F) blocked infection of unfractionated parasites in vitro and in vivo (5). These antibodies were isolated by adsorbing human Chagasic sera on a peptide C-Sepharose column and performing elution with soluble peptide C (5). We found that affinity-purified peptide C antibodies were also very potent in neutralizing infection of Vero cells by TS^+ trypomastigotes (Fig. 4). This result suggests that TS^+ invasiveness is caused, at least in part, by the *trans*-sialidase marker.

We also attempted to determine whether peptide C antibodies would block TS^- trypomastigote invasion, but we could not get meaningful results because of the intrinsically low degree of infection induced by the parasites bearing little or no *trans*-sialidase.

Rescue of TS^- trypomastigote invasion phenotype by exogenous *trans*-sialidase. In face of the high rate of TS^+ parasite

FIG. 3. Invasive properties of trypomastigote populations. Assays to determine attachment to (A) and penetration (B) and infection (C) of monkey Vero cells were performed as described in Materials and Methods. Similar results were obtained with mink lung epithelial cells. Tryps, trypomastigotes. ▲, unfractionated population; ■, TS⁺; □, TS⁻. Error bars indicate standard deviations.

entry into mammalian cells, a simple hypothesis to explain the opposite behavior of their TS⁻ counterparts should invoke the very absence of *trans*-sialidase in the noninvasive parasites. To determine whether this is true, we infected epithelial cells and fibroblasts with TS⁻ trypomastigotes in the presence of various concentrations of purified *trans*-sialidase to see if the exogenous enzyme would render the TS⁻ parasites invasive.

Indeed, introduction of low concentrations of purified enzyme into suspensions of TS⁻ trypomastigotes upped invasion by more than 30-fold from a control level of 0.8% (absence of exogenous enzyme) to ~25% (optimum dose of exogenous enzyme) (Fig. 5A). Such dramatic rescue of the TS⁻ invasion phenotype by exogenous *trans*-sialidase was reproducible in more than 15 other independent experiments.

In addition to making TS⁻ parasites invasive, exogenous *trans*-sialidase augmented infection by unfractionated and TS⁺ parasites, although by a relatively slight 5-fold (from 4 to 21%) and 1.7-fold (from 16 to 27%), respectively (Fig. 5A). These results further indicate that the *trans*-sialidase is an upregulator of *T. cruzi* infection.

Upregulation by the *trans*-sialidase was specific for the *T. cruzi* enzyme because the sialidases from the bacterium *Vibrio cholerae* (Fig. 5B) and Newcastle disease virus (data not shown) reduced, rather than enhanced, infection by TS⁺ and unfractionated parasites. The reduction of invasion brought about by bacterial and viral sialidases was in keeping with published results (5, 14).

Expression of other invasion factors in TS⁺ and TS⁻ parasites. One might explain the contrasting invasiveness of TS⁺ and TS⁻ trypomastigotes by the differential expression not only of *trans*-sialidase but also of other, as-yet-unidentified phenotypic markers of *T. cruzi*. We therefore looked for the expression of several determinants of invasion on the two trypomastigote subsets. TGF- β -like activity was determined by transcriptional activation of a TGF- β -responsive reporter stably transfecting mink epithelial cells (15). Unlike the situation with *trans*-sialidase expression in the various populations, which was polar (Fig. 2), differences in expression of the TGF- β -like factor in TS⁺, TS⁻, and unfractionated parasites were not readily apparent (Fig. 6). Similarly, we could not find any

difference in the expressions of penetrin and of a sialic acid epitope (ssp3 [25]) in the various trypanosome populations when they were assayed by immunofluorescence (data not shown). Although these results do not exclude the possibility of a unique (and so far unidentified) molecular marker in the TS⁻ parasites, they further emphasize the importance of the *trans*-sialidase in *T. cruzi* invasion.

High-level virulence of TS⁺ trypomastigotes for mice. To determine whether the enhanced invasiveness of TS⁺ trypanosomes for cultured cells would be reflected in actual infection of animals, we compared the courses of infection of BALB/c mice whose peritoneal cavities were injected with TS⁺, TS⁻,

FIG. 4. Inhibition of TS⁺ trypomastigote infection by human *trans*-sialidase antibodies. Vero cells were infected with TS⁺ trypomastigotes in the presence of the indicated concentrations of peptide C antibodies. These antibodies were the same as those used in a previous study (13). □, anti-peptide C IgG; ■, normal IgG. Error bars indicate standard deviations.

FIG. 5. Rescue of TS⁻ trypomastigote invasion by exogenous *trans*-sialidase. (A) Trypanosome sialidase. TS⁻ (□), TS⁺ (■), and unfractionated (▲) trypomastigotes were allowed to infect Vero cells for 1 h at 37°C in the presence of the indicated concentrations of purified *trans*-sialidase. After washing, infection proceeded for 3 days. Results are plotted as increase over control levels (absence of exogenous enzyme). (B) Bacterial sialidase. The assay was as described for panel A except the sialidase was from *V. cholerae*. Results are plotted as fold decrease in infection. Results with the TS⁻ subset are not shown because the level of infection was too low (0.8%) prior to the treatment with bacterial sialidase to give statistically significant results.

and unfractionated parasites. Infection was monitored by measuring parasitemia and mortality. Mimicking the results obtained *in vitro*, mice inoculated with the TS⁺ subset developed a higher level of parasitemia and died earlier than mice infected with unfractionated trypanosomes (Fig. 7). In contrast, TS⁻ trypomastigotes gave a low-level, transient parasitemia and did not cause death at an inoculum that with TS⁺ and unfractionated parasites produced 100% mortality (5,000 trypanosomes per mouse) (Fig. 7). Similar results were obtained by peritoneal injection in the inoculum range of 1,000 to 6,000 trypanosomes per mouse.

DISCUSSION

The expression of *trans*-sialidase in *T. cruzi* trypomastigotes provided a unique opportunity to explore a possible correlation of parasite populations with invasion. It also offered the chance to test the functional correlation of invasion of a given trypanosome population with the defining phenotypic marker. We found many years ago that trypomastigotes inhabiting the extracellular milieu of cell cultures and infected mice comprise two populations characterized by the presence (TS⁺) and absence (TS⁻) of *trans*-sialidase (4, 21). Given that *trans*-sialidase is a surface-bound and secreted enzyme implicated in attachment and invasion of *T. cruzi* (19), we raised the possibility that the TS⁺ and TS⁻ populations might play distinguishing roles in parasite-host interactions. However, we couldn't directly test this hypothesis earlier because of difficulties in obtaining pure populations of viable TS⁺ and TS⁻ parasites.

We now report that TS⁺ and TS⁻ parasites can be rapidly and reproducibly separated from each other by exposing them to magnetic beads coated with the *trans*-sialidase MAb TCN-2 (21). Immunomagnetic beads are widely used to isolate lym-

FIG. 6. Transcriptional activation of the plasminogen activator inhibitor 1 (PAI-1) promoter by *T. cruzi* populations. Mink epithelial cell monolayers, stably transfected with a plasmid carrying the luciferase gene under control of the PAI-1 promoter, were infected with the indicated amounts of *T. cruzi* for ~14 h, lysed in nonionic detergent, and assayed for luciferase (reporter) activity. ■, TS⁺; □, TS⁻; ▲, unfractionated; △, epimastigotes. Error bars indicate standard deviations.

FIG. 7. Time course of infection of mice with trypanomastigote populations. A group of five BALB/c mice (8 weeks old) were inoculated intraperitoneally with 5,000 TS⁺ (■), unfractionated (▲), and TS⁻ (□) parasites. Parasitemia (A) and survival (B) were monitored at the indicated times. This experiment was repeated three times with similar results.

phocytes (12), endothelial cells (10), and other cell types (29). We adapted the immunomagnetic bead procedure to isolate two trypanosome populations distinguished from one another by the expression of surface *trans*-sialidase. One advantage of our immune selection is that the trypanosome populations are purified in one step: the TS⁺ population by positive selection and the TS⁻ population by negative selection (Fig. 1). In most other cases, such as the separation of lymphocyte populations (12) and of human basophils from other leukocytes (1), the population of interest is usually obtained by negative selection

because of difficulties in eluting cells adhering to the beads. In our study, adherent parasites were readily detached from the beads under physiological conditions with a synthetic peptide epitope of TCN-2 (Fig. 1) (22). The positively and negatively selected trypanosomes were determined to be TS⁺ and TS⁻, respectively, by immunoblotting, immunofluorescence, and enzyme activity measurements (Fig. 2).

It should be noted that the positively selected TS⁺ population is consistently isolated in near-100% purity, as very few, if any, amastigotes and host cell debris are retained nonspecifically by the beads. However, suspensions of TS⁻ parasites may contain such contaminants if they were present in the original trypanomastigote suspension. Therefore, if TS⁺ and TS⁻ parasites are to be purified in one step, care should be taken to start with trypanomastigotes devoid of contaminants, a state which is usually accomplished with parasites harvested from Vero cell monolayers 5 to 7 days after the start of infection. TS⁺ parasites are readily isolated at a high level of purity not only from cell culture overlays but also from the blood of infected mice (often by simply incubating infected blood with TCN-2-coated beads), in which case the TS⁻ bloodstream trypanomastigotes will surely be mixed with blood cells (19a).

Functional analysis revealed a marked dichotomy in the invasive properties of the TS⁺ and TS⁻ populations both in vitro and in vivo. TS⁺ parasites adhered to culture cells better than unfractionated and TS⁻ parasites did (Fig. 3). TS⁺ parasites were also much more efficient in entering and thriving in host cells than the other parasite types (Fig. 3). These findings were confirmed in vivo, as TS⁺ parasites were highly virulent compared with unfractionated and TS⁻ parasites (Fig. 7). The high level of invasiveness of TS⁺ parasites is most likely a consequence of *trans*-sialidase expression, for antibodies against the enzyme blocked invasion (Fig. 4).

In contrast to the TS⁺ population, the TS⁻ population invaded cells very poorly both in vitro (Fig. 3) and in vivo (Fig. 7). Surprisingly, they attached to cell monolayers relatively well, though somewhat more poorly than did TS⁺ parasites (Fig. 3). Attachment of TS⁻ parasites is explained by their penetrin content, which was judged to be similar to that of TS⁺ parasites on the basis of immunofluorescence assays. Penetrin is a *T. cruzi* protein that mediates attachment by a pathway (heparan sulfate receptors) distinct from the *trans*-sialidase pathway (sialyl epitopes) (9, 16).

In addition to penetrin, TS⁻ parasites contained other factors that contribute to invasion, namely, sialylated epitopes (25) and TGF- β -like activity (15), at levels indistinguishable from those in the TS⁺ counterparts. Given that TS⁻ parasites are noninvasive, it is surprising that they contained at least three invasion factors at levels similar to those in the highly invasive TS⁺ trypanomastigotes.

We don't know yet why the TS⁻ parasites are such poor invaders, but a major contributing factor may very well be their poor expression of *trans*-sialidase. Accordingly, the addition of purified *trans*-sialidase at low concentrations (nanogram-per-milliliter range) to suspensions of TS⁻ parasites restored invasion in a dramatic fashion (Fig. 5A). The magnitude of the *trans*-sialidase-induced enhancement of TS⁻ invasion (~30-fold) is best appreciated when contrasted with the alterations in *T. cruzi* penetration brought about by drugs affecting lysosomal movement (28) or by deficiency in surface sialyl epitopes (14), which were by no more than three- to fourfold!

Rescue of the invasion phenotype was specific for the *trans*-sialidase, for bacterial and viral sialidases reduced, rather than enhanced, infection. The contrary action of bacterial and viral sialidases seems to exclude the removal of sialylated epitopes

from the parasites or host cells as a mechanism for the agonistic effect of *trans*-sialidase in invasion (Fig. 5B).

It is interesting that while *trans*-sialidase at low concentrations (nanogram-per-milliliter range) is a potent agonist of *T. cruzi* invasion, a much higher concentration (microgram-per-milliliter range) had the opposite effect (reference 14 and data not shown). Blocking of invasion by relatively high *trans*-sialidase concentrations was interpreted to mean competitive inhibition of trypanosome binding to sialyl epitope receptors on the surface of the host cell (14). If *trans*-sialidase at high doses is an antagonist of invasion, possibly at the level of attachment, how come low concentrations of the enzyme do not block but instead enhance invasion so profoundly? The concentrations of *trans*-sialidase causing enhancement of invasion (nanogram-per-milliliter range) are most likely too low to competitively block attachment of one cell (host) to another (trypanosome). In general, effective inhibition of cell-cell adhesion by large (molecular mass, ~50 to 200 kDa) ligands (8) or by antibodies to surface receptors (7) requires the soluble inhibitors to be in the microgram-per-milliliter or even milligram-per-milliliter range. This concentration range is also valid for the inhibition of many types of microbe-host cell interactions. For example, rhinovirus invasion of HeLa cells is inhibited by soluble intercellular adhesion molecule-1 (ICAM-1) (molecular mass, 82 kDa) at 1 to 10 μ g/ml (13), a concentration range analogous to that required for the *trans*-sialidase to inhibit *T. cruzi*-cell adhesion (molecular mass, 120 to 200 kDa) (14, 20, 21).

An explanation for the agonist effect of the *trans*-sialidase might depend on the location of the exogenous enzyme. The enzyme may have to be located at the surface of the TS⁻ parasites to render them TS⁺. This would promote parasite attachment and perhaps entry into cells. However, we think this is an unlikely explanation because TS⁻ parasites are poor invaders despite their ability to attach to cells relatively well in the absence or near absence of *trans*-sialidase.

Yet another possibility would be for the added *trans*-sialidase, whether localizing on the surface of the parasites or remaining in solution, or both, to activate the host cells and make them more susceptible to invasion, a hypothesis in line with current concepts of *T. cruzi* invasion (3, 15).

Our finding that the TS⁻ subset represents a major population of noninvasive trypomastigotes may explain some data in the literature regarding *T. cruzi* populations. For example, de Titto et al. isolated a major population of "nonpenetrating" trypomastigotes by exposing mouse fibroblast L929 cells to bloodstream parasites for 18 h to "filter" the invasive forms through the cell monolayer (6). After the nonpenetrating parasites were transferred several times to fresh L929 monolayers, a population of trypomastigotes that infected cells in vitro and in vivo very weakly compared with unfractionated parasites was obtained. Thus, the nonpenetrating population of swimming trypomastigotes behaved like our TS⁻ subset, while the population filtered through the fibroblast monolayer, which cannot be isolated by the protocol of de Titto et al. (6), should correspond to the TS⁺ phenotype.

We previously proposed a model of *T. cruzi* invasion (15) which starts with trypomastigotes attaching to cells through *trans*-sialidase or sialic acid and penetrin or heparan sulfate pathways. The host cells become permissive of trypanosome entry after undergoing several biochemical alterations, including activation of the TGF- β signaling pathway (15), a rise in intracellular Ca²⁺ levels, and cytoskeletal rearrangement (3). We now modify this model to limit invasion to a small population of extracellular trypomastigotes carrying the phenotypic marker *trans*-sialidase. The *trans*-sialidase is not only a marker for the invaders but also a driving force for penetration, as the

TS⁻ population, which lacks the enzyme, is extremely inefficient in infecting cells. Most important, TS⁻ parasites could change into organisms with an invasive phenotype in vitro by simple reconstitution with exogenous *trans*-sialidase. Whether such rescue of the invasion phenotype in the TS⁻ population occurs in vivo remains to be determined.

ACKNOWLEDGMENTS

We thank Victor Nussenzweig for MAb 3C9, Marina Chuenkova for help in the experiments with mice, and Jacqueline Sharon for comments on the manuscript.

This work was supported by National Institute of Health grants PO1 AI38084 and AI18102.

REFERENCES

1. Bjerke, T., S. Nielsen, J. Helgestad, B. W. Nielsen, and P. O. Schioltz. 1993. Purification of human blood basophils by negative selection using immunomagnetic beads. *J. Immunol. Methods* **157**:49–56.
2. Brener, Z. 1973. The biology of *Trypanosoma cruzi*. *Annu. Rev. Microbiol.* **27**:349–381.
3. Burleigh, B. A., and N. Andrews. 1995. The mechanism of *Trypanosoma cruzi* invasion of mammalian cells. *Annu. Rev. Microbiol.* **49**:175–200.
4. Cavaleco, R., and M. E. A. Pereira. 1988. Antibody to *Trypanosoma cruzi* neuraminidase enhances infection in vitro and identifies a subpopulation of trypomastigotes. *J. Immunol.* **140**:617–625.
5. Chuenkova, M., and M. E. A. Pereira. 1995. *Trypanosoma cruzi* *trans*-sialidase: enhancement of virulence in a murine model of Chagas' disease. *J. Exp. Med.* **181**:1693–1703.
6. de Titto, E. H., D. Israelski, and F. G. Araujo. 1987. Circulating *Trypanosoma cruzi* from the same cloned population show differences in the ability to infect cells and to cause lethal infection in mice. *Experientia* **43**:1227–1229.
7. Geng, J.-G., M. P. Bevilacqua, K. L. Moore, T. M. McIntyre, S. M. Prescott, J. M. Kim, G. A. Bliss, G. A. Zimmerman, and R. P. McEver. 1990. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature (London)* **343**:757–760.
8. Gabel, L. B., S. D. Rosen, and G. R. Martin. 1979. Teratocarcinoma stem cells have a cell surface carbohydrate-binding component implicated in cell-cell adhesion. *Cell* **17**:477–484.
9. Herrera, E. M., M. Ming, E. Ortega-Barria, and M. E. A. Pereira. 1994. Mediation of *Trypanosoma cruzi* invasion by heparan sulfate receptors on host cells and penetrin counter-receptors on the trypanosomes. *Mol. Biochem. Parasitol.* **65**:73–83.
10. Jackson, C. J., P. K. Garbett, B. Nissen, and L. Schreiber. 1992. Binding of human endothelium to *Ulex europaeus* lectin I-coated Dynabeads: application to the isolation of microvascular endothelium. *J. Cell Sci.* **96**:257–262.
11. Janeway, C. A., Jr., and P. Travers. 1994. Immunobiology: the immune system in health and disease, p. 1:1–2:67. Current Biology/Garland Publishing, New York.
12. Manyonda, I. T., A. J. Soltys, and F. C. Hay. 1992. A critical evaluation of the magnetic cell sorter and its use in the positive and negative selection of CD45RO⁺ cells. *J. Immunol. Methods* **149**:1–10.
13. Marlin, S. D., D. A. Staution, T. A. Springer, C. Stratowa, W. Sommergruber, and V. J. Merluzzi. 1990. A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. *Nature (London)* **344**:70–72.
14. Ming, M., M. Chuenkova, E. Ortega-Barria, and M. E. A. Pereira. 1993. Mediation of *Trypanosoma cruzi* invasion by sialic acid on the host cell and *trans*-sialidase on the trypanosomes. *Mol. Biochem. Parasitol.* **59**:243–252.
15. Ming, M., M. E. Ewen, and M. E. A. Pereira. 1995. *Trypanosoma* invasion of mammalian cells requires activation of the TGFB signaling pathway. *Cell* **82**:287–296.
16. Ortega-Barria, E., and M. E. A. Pereira. 1991. A novel *T. cruzi* heparin-binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. *Cell* **67**:411–421.
17. Parodi, A. J., G. D. Pollevick, M. Mautner, A. Buschiazio, D. O. Sanchez, and A. C. Frasch. 1992. Identification of the gene(s) coding for the *trans*-sialidase of *Trypanosoma cruzi*. *EMBO J.* **11**:1705–1710.
18. Pereira, M. E. A. 1983. A developmentally regulated neuraminidase activity in *Trypanosoma cruzi*. *Science* **219**:1444.
19. Pereira, M. E. A. 1994. Intracytosolic survival of *Trypanosoma cruzi*, p. 305–334. In D. G. Russell (ed.), *Bailliere's clinical infectious diseases*. Bailliere Tindall, Philadelphia.
- 19a. Pereira, M. E. A. Unpublished data.
20. Pereira, M. E. A., J. S. Mejia, E. Ortega-Barria, D. Matzilevich, and R. P. Prioli. 1991. The *Trypanosoma cruzi* neuraminidase contains sequences similar to bacterial neuraminidases, YWTD repeats of the low density lipoprotein receptor, and type III modules of fibronectin. *J. Exp. Med.* **174**:179–191.
21. Prioli, R. P., J. S. Mejia, and M. E. A. Pereira. 1990. Monoclonal antibodies against *Trypanosoma cruzi* neuraminidase reveal enzyme polymorphism, rec-

- ognize a subset of trypomastigotes, and enhance infection in vitro. J. Immunol. **144**:4384–4391.
22. Prioli, R. P., E. Ortega-Barria, J. S. Mejia, and M. E. A. Pereira. 1992. Mapping of a B cell epitope present in the neuraminidase of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. **52**:85–96.
 23. Rosenberg, I. A., R. P. Prioli, J. S. Mejia, and M. E. A. Pereira. 1991. Differential expression of *Trypanosoma cruzi* neuraminidase in intra- and extracellular trypomastigotes. Infect. Immun. **59**:464–466.
 24. Schenkman, S., D. Eichinger, M. E. A. Pereira, and V. Nussenzweig. 1994. Structural and functional properties of trypanosoma trans-sialidase. Annu. Rev. Microbiol. **48**:499–532.
 25. Schenkman, S., M.-S. Jiang, G. H. Hart, and V. Nussenzweig. 1991. A novel cell surface trans-sialidase of *Trypanosoma cruzi* generates a stage specific epitope required for invasion of mammalian cells. Cell **65**:1117–1125.
 26. Schenkman, S., L. Pontes de Carvalho, and V. Nussenzweig. 1992. *Trypanosoma cruzi* trans-sialidase and neuraminidase activities can be mediated by the same enzyme. J. Exp. Med. **175**:567–575.
 27. Scudder, P., J. P. Doom, M. Chuenkova, I. D. Manger, and M. E. A. Pereira. 1993. Enzymatic characterization of the β -D-galactoside α 2,3-trans-sialidase from *Trypanosoma cruzi*. J. Biol. Chem. **268**:9886–9891.
 28. Tardieux, I., P. Webster, J. Ravesloot, W. Boron, J. A. Lunn, J. E. Heuser, and N. W. Andrews. 1992. Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells. Cell **71**:1117–1130.
 29. Vremec, D., M. Zorbas, R. Scollay, D. J. Saunders, C. F. Ardovin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. J. Exp. Med. **176**:47–58.

Editor: J. M. Mansfield