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Trypanosoma cruzi trypomastigote clones differentially express a parasite cell adhesion molecule

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We have cloned blood trypomastigotes from infected mice and found that *Trypanosoma cruzi* strains are composed of heterogeneous populations that dramatically vary (more than 100 fold) in their abilities to attach to and enter rat heart myoblasts. Trypomastigote clones were distinctively separated into highly and weakly infective groups presenting higher and lower rates of attachment to myoblasts, respectively. Each trypomastigote clone maintained the same profile of attachment and internalization into heart myoblasts when tested at different periods of time. This pattern did not change when the parasites were incubated in fresh medium before being exposed to heart myoblasts. Highly and weakly infective clones show differences at the cell surface level, particularly with regard to a 83 kDa glycoprotein. We have identified this 83 kDa glycoprotein as the parasite membrane ligand that specifically binds to rat heart myoblasts. The binding of the biotinylated 83 kDa to myoblasts is inhibited by cold excess in Western blots, as indicated by laser densitometry. In addition, the specific binding of this molecule to myoblasts is saturable and is greater in highly than in weakly infective trypomastigote clones. Highly invasive trypomastigote clones express this glycoprotein in more abundance on their surface than weakly infective trypomastigote clones. These results indicate that the 83 kDa glycoprotein present on the surface of *T. cruzi* trypomastigotes mediates the attachment of the parasite to heart myoblasts.

Key words: *Trypanosoma cruzi*; 83 kDa glycoprotein; Parasite cell adhesion molecule; Highly and weakly infective clones; Differential parasite binding; Host cell infection

Introduction

Trypanosoma cruzi, the protozoan that causes Chagas' disease, which is a major health problem in South and Central America, requires an attachment step to penetrate mammalian cells [1]. Since the parasite only multiplies intracellularly, cell invasion is a vital step in its cycle. Therefore, a thorough understanding of the mechanisms of parasite attachment and penetration of host cells is essential to the development of effective means to prevent the disease. The identification of *T. cruzi* surface molecules mediating the interaction between the parasites and the host cells is an obligatory step in the understanding of this overall process.

It has been suggested that membrane components participate in this interaction, since a crude membrane preparation is able to partially inhibit parasite–host cell attachment [2]. In addition several observations from different laboratories have suggested that sugar moieties of glycoproteins on the cell surface of parasites could be involved in this process, since removal of these moieties and inhibition of glycoprotein biosynthesis or processing can affect the attachment of the parasite to different host cells [3–8]. However, specific par-

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Abbreviations: A-HRP, avidin horseradish peroxidase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] 1-propane-sulfonate; DMEM, Dulbecco's modified minimal essential medium; DMEM-BSA, DMEM containing 1% bovine serum albumin; DMEMS, DMEM containing 10% heat inactivated fetal bovine serum; HBSS, Hanks' balanced salt solution; PAS, periodic acid Schiff; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; NHS-biotin, *N*-hydroxysuccinimide-biotin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLCK, *N*-*p*-tosyl-L-lysine-chloromethyl ketone.

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asite molecules that mediate attachment of parasites to muscle cells have not been identified.

We have observed that blood trypomastigotes (Tulahuen strain) isolated at different times from infected animals differ markedly in their degree of association with rat heart myoblasts using an in vitro parasite–host cell association assay over a period of time [3–6,8]. This suggests that trypomastigote forms found in the blood of infected mice are composed of heterogeneous populations with different degrees of cellular invasiveness since we have repeatedly observed this phenomenon using standardized conditions [3–6]. In order to test this hypothesis and establish a working model that might rapidly reveal parasite ligands responsible for the binding of the parasite to host cells, we cloned blood trypomastigotes and studied the ability of trypomastigote clones to attach and invade rat heart myoblasts, correlating the parasite surface protein profiles with their ability to attach to host cells.

Materials and Methods

Parasites. The Tulahuen strain of *T. cruzi* was used in most of the experiments. The Y strain of *T. cruzi* was also used. Parasites were maintained by intraperitoneal inoculation of 1×10^5 blood trypomastigotes in 4-week-old Crl:CD-1(ICR)BR, Swiss mice purchased from Charles River Laboratories (Raleigh, NC) [9,10]. Mice were bled under sterile conditions, by cardiac puncture, and blood trypomastigotes were isolated by centrifugation of the blood over Ficoll-Hypaque followed by chromatography on diethylaminoethyl-cellulose columns [11]. Culture trypomastigote clones were obtained by infecting rat heart myoblasts with blood trypomastigotes as described above.

Trypomastigote cloning. Isolated blood trypomastigotes were washed by centrifugation with Dulbecco's modified Minimal Essential Medium (DMEM; GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated (56°C, 1 h) fetal bovine serum (GIBCO), penicillin (100 units ml^{-1}) and streptomycin (100 $\mu g\ ml^{-1}$) (DMEMS), and resuspended in the same medium. Trypomastigote cloning was carried out by limiting dilution. Blood trypomastigotes were resuspended at a concentration of 20 organisms ml^{-1} and 50 μl aliquots containing 1 parasite were placed in 96-well flat bottom tissue culture plates (Corning, NY). Each well contained a full-grown rat heart myoblast (American Type Culture Collection) monolayer. Cells and parasites were incubated at 37°C in a 10% CO_2-in-air incubator for 24 h. After this time the cultures were washed with DMEM; 200 μl of fresh DMEMS was added and the cultures were incubated in the same conditions as described above. After two weeks the first clones were detected by the appearance of trypomastigotes in the culture supernatants. Trypomastigote clones were expanded in rat heart myoblast monolayers and recloned again as described above by adding 50 μl aliquots containing 0.1 parasites per monolayer. Recloned trypomastigotes were numbered and used to test their ability to attach to and penetrate into rat heart myoblasts as described in the next section.

Attachment and internalization of trypomastigote clones into rat heart myoblasts. To measure attachment and internalization, the basic assay previously used to measure association of *T. cruzi* trypomastigotes with rat heart myoblasts [3–6] was modified. Rat heart myoblast monolayers were prepared in microwells of Teflon slides (3 mm diameter, Cel-Line, New Jersey, NJ) and were allowed to grow in DMEMS at 37°C as described above for two days. Trypomastigote clones obtained from rat heart myoblasts were washed twice in DMEM at $800 \times g$ for 20 min. The parasites were resuspended in DMEM containing 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) (DMEM-BSA) at a concentration of 1×10^7 organisms ml^{-1}. 20 μl of this parasite suspension were added to the microwells of Teflon-coated slides. The cultures were incubated at 37°C for 2 h. Under these conditions the parasite attaches to the host cells and minimal internalization occurs (ref. 12 and Villalta, F., unpublished observations). After washing off the non-bound organisms with phosphate buffered saline, pH 7.2 (PBS), the monolayers were fixed with 2.5% glutaraldehyde in PBS and stained with Giemsa. The number of trypomastigotes attached per 100 cells and the percentage of myoblasts containing

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attached parasites were evaluated microscopically by screening no less than 200 cells per well [3–6]. For parasite internalization assays, the above protocol was modified by adding fresh DMEM-BSA after washing off the non-bound trypomastigotes and allowing incubation for an additional 2 h. At the end of this time, the extracellular parasites that remained bound to the myoblasts were lysed by a 2 min hypotonic pulse [12], and the monolayers were fixed as described above. In some experiments, the internalized parasites were allowed to grow for 72 h and this was accomplished by adding fresh DMEMS to the cultures after the hypotonic pulse. After this time, the monolayers were fixed, stained and the number of intracellular parasites per cell and the percentage of infected cells were microscopically determined as described above.

Biotinylation of trypomastigotes. Cell surface proteins of highly and weakly infective clones were biotinylated by adding a 2.6 mM final concentration of *N*-hydroxysuccinimide biotin (NHS-Biotin) (BioRad, Richmond, CA), dissolved in dimethyl formamide to 5×10^8 parasites resuspended in PBS for 15 min at room temperature. Parasites remained motile and morphologically similar throughout the procedure as observed by optical microscopy. Parasites were then washed three times with cold PBS, and solubilized with 0.8% 3-[(3-cholamidopropyl)-dimethylammonio] 1-propane-sulfonate (CHAPS) [13] in PBS in the presence of protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM *p*-tosyl-L-lysinechloromethylketone (TLCK), and 2.8 μg ml^{-1} aprotinin]. Solubilized parasites were centrifuged at $13000 \times g$ for 5 min at 4°C to remove debris. In experiments where the effect of biotinylation of trypomastigote clones on the ability of the parasite to attach to and enter into rat heart myoblasts was evaluated, parasites were labeled as described in this section and exposed to rat heart myoblasts as described in detail above.

Western blots. 5 μg protein of biotinylated parasite samples from highly and weakly infective clones, obtained as described above, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% acrylamide gel, in the presence or absence of β-mercaptoethanol according to Laemmli [14] in a mini PROTEAN II cell (BioRad). Gels were then electroblotted onto 0.45 μm nitrocellulose membranes (BioRad) for 1 h at 4°C in a mini Trans Blot cell (BioRad). Biotinylated molecular weight markers (BioRad) were included in the gels to determine the relative molecular weight of parasite samples. After the transfer was complete the gels were stained with silver stain (BioRad) to check for the efficiency of transfer. A complete and efficient transfer had been achieved using these conditions. Nitrocellulose membranes were then stained with avidin-horseradish peroxidase (A-HRP; BioRad), and developed with 4-chloro-1-naphthol/H_2O_2. In some instances gels were stained with periodic acid-Schiff (PAS) [15], and silver-Coomassie blue [16].

Interaction of trypomastigote surface proteins with rat heart myoblasts. To identify which parasite component, resolved by SDS-PAGE, bound to rat heart myoblasts we modified the technique used by Baseman and Hayes [17] to identify the ligands of *Treponema pallidum* that bind to epithelial cells. Two types of trypomastigote clones, highly (MMC 20A) and weakly (MMC 151D) infective, were selected for these studies. Biotinylated trypomastigote clones were solubilized with 0.8% CHAPS in the presence of protease inhibitors as described above. After removing the detergent by extensve dialysis against PBS at 4°C, 1 mg biotinylated parasite proteins from each clone was incubated with monolayers of live heart myoblasts (2×10^6 cells per 60 mm culture dish) for 4 h at 4°C to avoid ligand internalization. Unbound parasite material was removed by very gently washing the cell monolayer with cold PBS to avoid removing cells in the monolayer. Myoblasts containing the bound parasite attachment molecule were then solubilized with 0.8% CHAPS [13] and the same protease inhibitors as described above. Samples were centrifuged at $13000 \times g$ at 4°C, dialyzed against water at 4°C, and lyophilized. 120 μg protein of each sample were then subjected to SDS-PAGE, blotted onto nitrocellulose membranes, reacted with A-HRP and developed with 4-chloro-1-naphthol/H_2O_2.

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Laser scanning. Nitrocellulose membranes containing either blotted biotinylated surface proteins of *T. cruzi* trypomastigotes or the dissociated parasite adhesion molecule from rat heart myoblasts reacted with A-HRP were scanned with an LKB Ultroscan XL Laser densitometer.

Inhibition experiments in Western blots. Rat heart myoblasts (3×10^5 cells per well) in 24-well plates were pre-incubated with 200 μl of different concentrations of detergent-free CHAPS-solubilized preparations of unlabeled trypomastigotes from clone MMC 20A (ranging from 0.6 to 2.5 mg of protein) at 4°C for 4 h. After this period unbound parasite material was removed by washing and the monolayers were incubated with 200 μl of a constant concentration of solubilized biotinylated trypomastigotes from the same clone (90 μg) as described above. After extensive washing, the monolayers were solubilized with CHAPS and samples (14 μg protein) were resolved by SDS-PAGE, blotted onto nitrocellulose and stained with streptavidin horseradish peroxidase (Bio-Genex, Dublin, CA). Nitrocellulose membranes showing only the 83 kDa band were scanned with a laser densitometer. The percentage of inhibition was determined applying the following formula: $\% I = (1 - A/B) \times 100$, where B is the area of the 83 kDa band in the absence of unlabeled counterpart and A is the area of the 83 kDa band in the presence of different concentrations of unlabeled counterpart.

Radioiodination of trypomastigotes. Pure culture trypomastigotes (2×10^8 organisms) were radioiodinated with 2 mCi of $Na^{125}I$ (specific activity 17 Ci mg^{-1} I, ICN Radiochemicals, Irvine, CA) in Iodogen (Pierce Chemical Co., Rockford, IL) coated tubes [18]. Unbound radioactivity was removed by five washes with HBSS. Radioiodination of parasites under these conditions does not affect the motility, morphology and ability of trypomastigotes to attach to and internalize into myoblasts (Lima, M.F. and Villalta, F., unpublished observations).

Binding of solubilized ^{125}I-trypomastigotes to myoblasts. Comparative binding of solubilized ^{125}I-labeled highly and weakly infective trypomastigote clones to myoblasts was performed by adding in triplicate increasing concentrations of parasite material, starting with the same specific activity of 1.27×10^5 cpm (μg protein)$^{-1}$, from both clones, to myoblast monolayers (1×10^5 cells well^{-1}) in 96-well plates. Cultures were incubated for 4 h at 4°C. After removing parasite unbound material by gently washing the monolayers three times with HBSS, the cells were solubilized with 1% SDS in the presence of protease inhibitors as described above and the radioactivity in 30 μl aliquots was measured with a gamma counter. Nonspecific binding was determined by adding an excess of 30 times solubilized unlabeled trypomastigote clones respectively, to each well. Specific binding was determined by subtracting the nonspecific binding from the total binding. Aliquots (30 μg protein) of SDS solubilized myoblasts containing the bound radioiodinated parasite material were resolved by SDS-PAGE and autoradiographed.

Presentation of results and statistics. The results presented in the tables and figures of this paper are typically representative of three to four independent experiments with the same design. Differences were considered to be significant if $P < 0.05$ as determined by Student's t-test.

Results

Characterization of T. cruzi trypomastigote clones according to the ability to attach to and enter heart myoblasts. Results using clones obtained by infecting rat heart myoblasts with a limiting dilution of blood trypomastigotes indicate that Tulahuen strain trypomastigotes are composed of heterogeneous populations of parasites with different degrees of cellular invasiveness. The initial screening of the ability of the 187 clones obtained to invade myoblasts was done by allowing parasites to interact with the cells for 4 h at 37°C. It was found that clones could be distinctively separated into highly, average, and weakly infective groups. Fig. 1 shows dramatic differences (more than 100 fold) between selected trypomastigote clones in their ability to invade myoblasts. Because we wished to determine whether the clones found to be more invasive also had a higher abil-

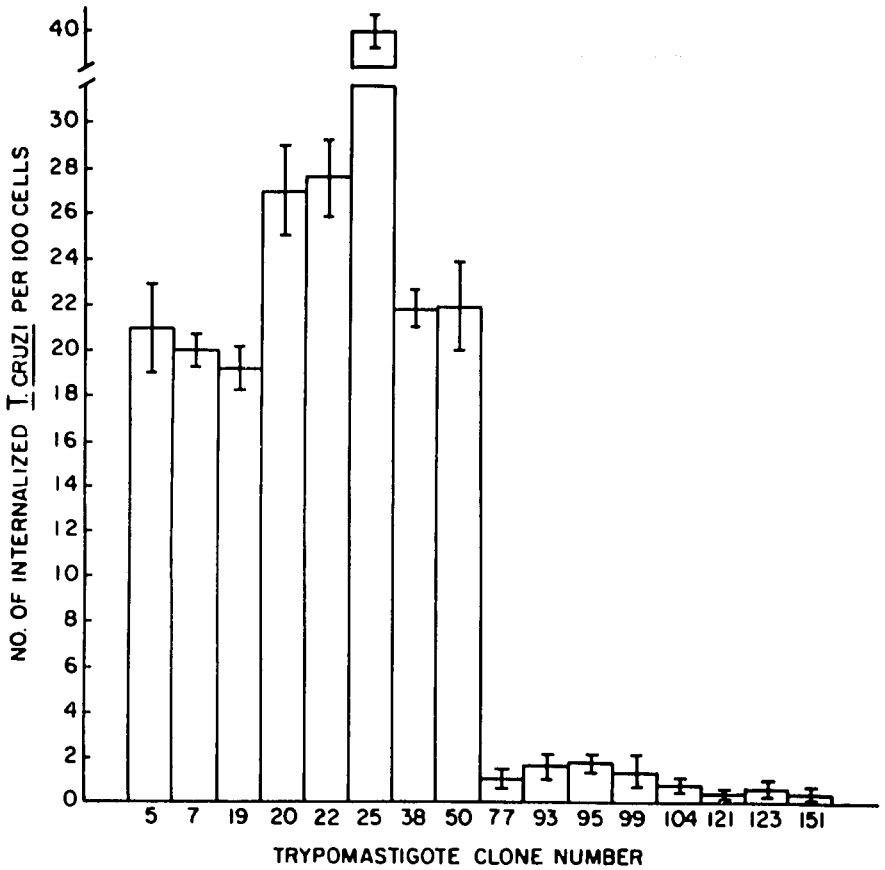

Fig. 1. Profile of internalization of selected *T. cruzi* trypomastigote clones into rat heart myoblasts. *T. cruzi* clones were grown in rat heart myoblasts and obtained as described in Materials and Methods. Parasites and cells (10:1 ratio) were allowed to interact for a total length of 4 h at 37°C. After removing the nonbound parasites and lysing the attached parasites the cultures were fixed and stained with Giemsa. No less than 200 cells were screened; the number of trypomastigotes internalized by cells was recorded. Each column represents the mean of triplicate determinations and the bars, one standard deviation. The differences between highly infective clones (5-50) and weakly infective clones (77-151) were statistically significant ($P < 0.005$).

ity to bind to cells, we devised experiments to minimize internalization by allowing parasites and myoblasts to interact at 37°C for only 2 h. Clones that we had classified as highly and weakly invasive presented higher and lower rates of attachment to host cells, respectively (Fig. 2). In addition, when fresh medium was added to rat heart myoblasts containing internalized parasites after 4 h of interaction and parasites were allowed to grow intracellularly for 72 h, the same clones presented similar rates of intracellular growth (Fig. 2). We observed that each trypomastigote clone maintains the same profile of attachment and internalization into host cells when tested at different times (Fig. 3), indicating that the clones are stable, and that they maintain their properties of attachment and invasion over time. We also found that the characteristics of attachment and invasiveness of highly and weakly infective trypomastigote clones did not change when parasites were incubated in fresh medium at 37°C for 1 h or more before being exposed to rat heart myoblasts (Table I). Y strain trypomastigotes were also cloned using the same methodology described above. Table II shows that two selected Y strain trypomastigote clones also differ dramatically in their ability to invade rat heart myoblasts.

Cell surface differences between highly and weakly infective trypomastigote clones. Since the attachment of *T. cruzi* trypomastigotes to host cells involves close contact between membranes of the

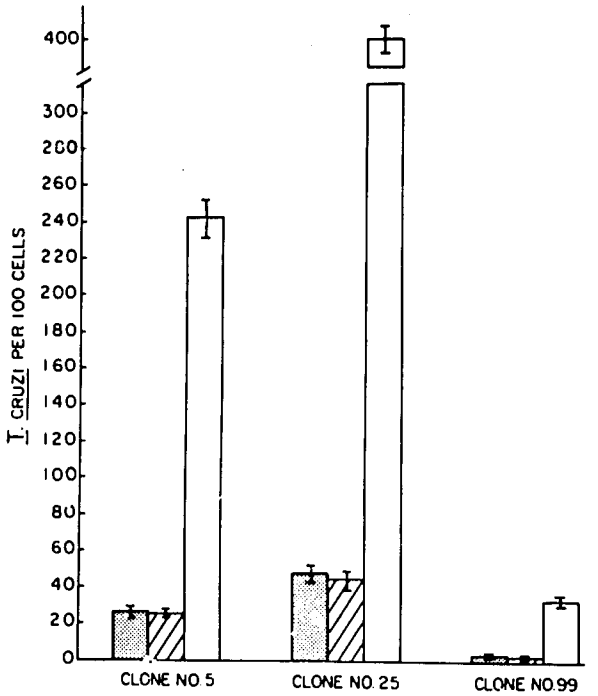

Fig. 2. Comparative binding, internalization and intracellular growth of three trypomastigote clones to monolayers of rat heart myoblasts. The interaction between parasite and rat heart myoblasts has been described in detail in Materials and Methods. Each column represents the mean of triplicate determinations and the bars one standard deviation. Stippled column: bound trypomastigotes, measured after 2 h of parasite–host cell interaction. Hatched column: internalized parasites measured after 4 h of parasite–host cell interaction. Open column: amastigote growth measured after 72 h parasite–host cell interaction.

parasite and the host cell, highly and weakly invasive clones may differ in their attachment and invasive abilities because of differences in their cell surface profiles. Western blots of biotinylated solubilized trypomastigote proteins indicated that surface components of highly and weakly infective trypomastigote clones differed with regard to some components. As shown in Fig. 4, right panel, lanes A and B, the major difference observed between these two types of clones is an 83 kDa band which is more abundant in highly (A) than weakly infective (B) trypomastigotes. When the nitrocellulose blots of the parasite surface protein profile from both clones were analyzed by laser densitometry (left panel, A and B) it was found that this 83 kDa band was 5.9 times more intense in highly than weakly infective clones. In addition, two minor bands of high molecular mass (178 and 159 kDa) are present in highly infective trypomastigote clones and are seen with much less intensity in the weakly infective trypomastigote clones. Conversely two minor bands of lower molecular mass (36 and 30 kDa) were more pronounced in the weakly infective than in the highly infective trypomastigote clones.

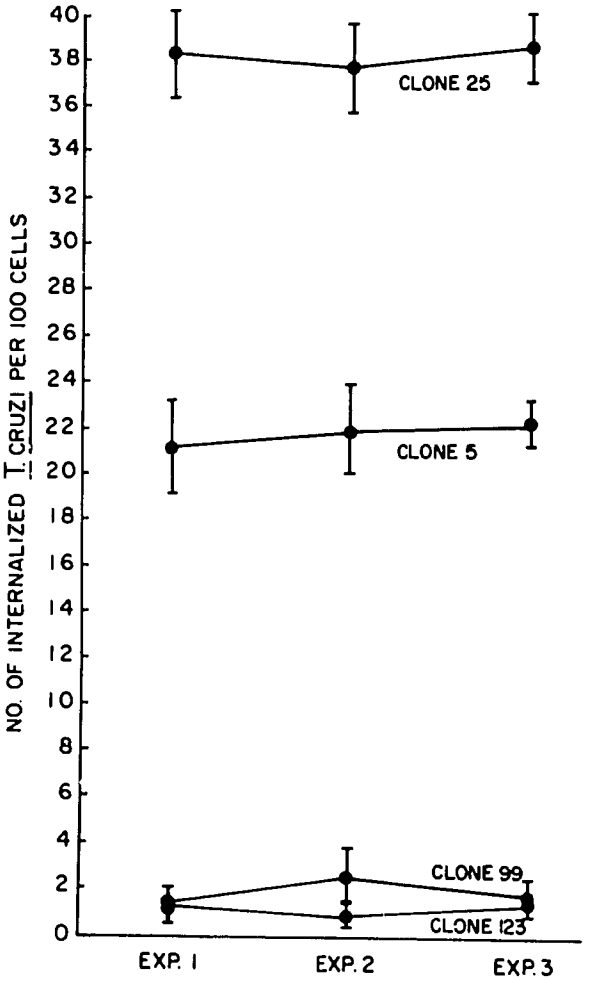

Fig. 3. Stability of invasiveness profiles of selected *T. cruzi* trypomastigote clones over time. The internalization of *T. cruzi* clones into host cells was evaluated as in Fig. 1. Experiments were performed independently at different times (15 day interval between experiments). Trypomastigote clones used in these experiments were passed several times in flasks containing myoblast monolayers, isolated and exposed to wells containing myoblasts. Each point represents the mean of triplicate determinations, and the bars one standard deviation.

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TABLE I

Invasiveness profiles of *T. cruzi* trypomastigote clones when pre-incubated in cell-free medium before being exposed to myoblasts

Clone number	Pre-incubation 37°C, 1 h	Percentage of cells containing internalized parasites	Number of parasites internalized per 100 cells
19	No	18.2 ± 1.3[a]	26.0 ± 1.4[a]
19	Yes	21.0 ± 1.6	29.6 ± 2.5
25	No	26.2 ± 3.9	43.2 ± 2.5
25	Yes	27.3 ± 1.6	47.3 ± 2.5
111	No	6.2 ± 1.1	7.2 ± 1.1
111	Yes	5.6 ± 0.7	6.8 ± 0.3

[a]The differences between test values and their controls were not statistically significant ($P < 0.05$).

Effect of biotinylation of surface proteins of trypomastigote clones on their ability to attach to myoblasts. We wished to determine if biotinylation of the surface protein of *T. cruzi* trypomastigotes would alter the capacity of the parasites to bind to host cells. As shown in Table III, neither biotinylation of parasites nor incubation of trypomastigotes with dimethyl formamide, the carrier of NHS-biotin, had any significant consequence on the attachment of highly and weakly infective clones to rat heart myoblasts. Therefore, this labeling procedure does not appear to affect the binding of parasite molecules to host cells. Similar results were obtained when trypomastigote surface proteins from both clones were radioiodinated (results not shown).

Identification of parasite surface proteins that bind to myoblasts. Since biotinylation of the cell surface proteins of highly and weakly infective clones did not modify their pattern of attachment to myoblasts, we exploited this fact to investigate which parasite surface component mediated the interaction between parasites and rat heart myoblasts using highly and weakly infective clones.

Detergent-free biotinylated parasite surface proteins from both types of clones were incubated with live myoblast monolayers for 4 h at 4°C to avoid ligand internalization. After removing unbound parasite material, and dissociating the adhesion molecule with CHAPS, the detergent was removed and the samples analyzed by SDS-PAGE, blotted onto nitrocellulose and stained with A-HRP. The results indicated that the 83 kDa component present in greater amounts on the cell surface of highly infective *T. cruzi* trypomastigotes is the component that binds to myoblasts (Fig. 4, right panel, lanes C and D). The binding of the 83 kDa band from highly infective trypomastigote clones to myoblasts is more pronounced (lane C) than the 83 kDa from weakly infective parasites (lane D). Scanning of nitrocellulose membranes by laser densitometry indicated that a single band from the cell surface proteins of both highly and weakly infective clones bound to myoblasts and that this band was present in greater quantities in highly than weakly infective clones (Fig. 4, left panel C and D). The specificity of the binding of the biotinylated 83 kDa molecule to myoblasts in Western blots was indicated by the concentration-dependent inhibitory effect of solubilized unlabeled trypomastigotes (Table IV). Laser densitometry analysis of nitrocellulose membranes containing the blotted 83 kDa showed that at the highest concentration

TABLE II

Y strain *T. cruzi* clones dramatically differ in their infectivity of rat heart myoblasts

Trypomastigote clone	Percentage of myoblasts containing internalized parasites	Number of parasites internalized per 100 cells
Y2	35.0 ± 2.6[a]	45.6 ± 3.3[a]
Y11	4.3 ± 1.6	4.1 ± 2.4

[a]The differences between both clones were statistically significant ($P < 0.005$).

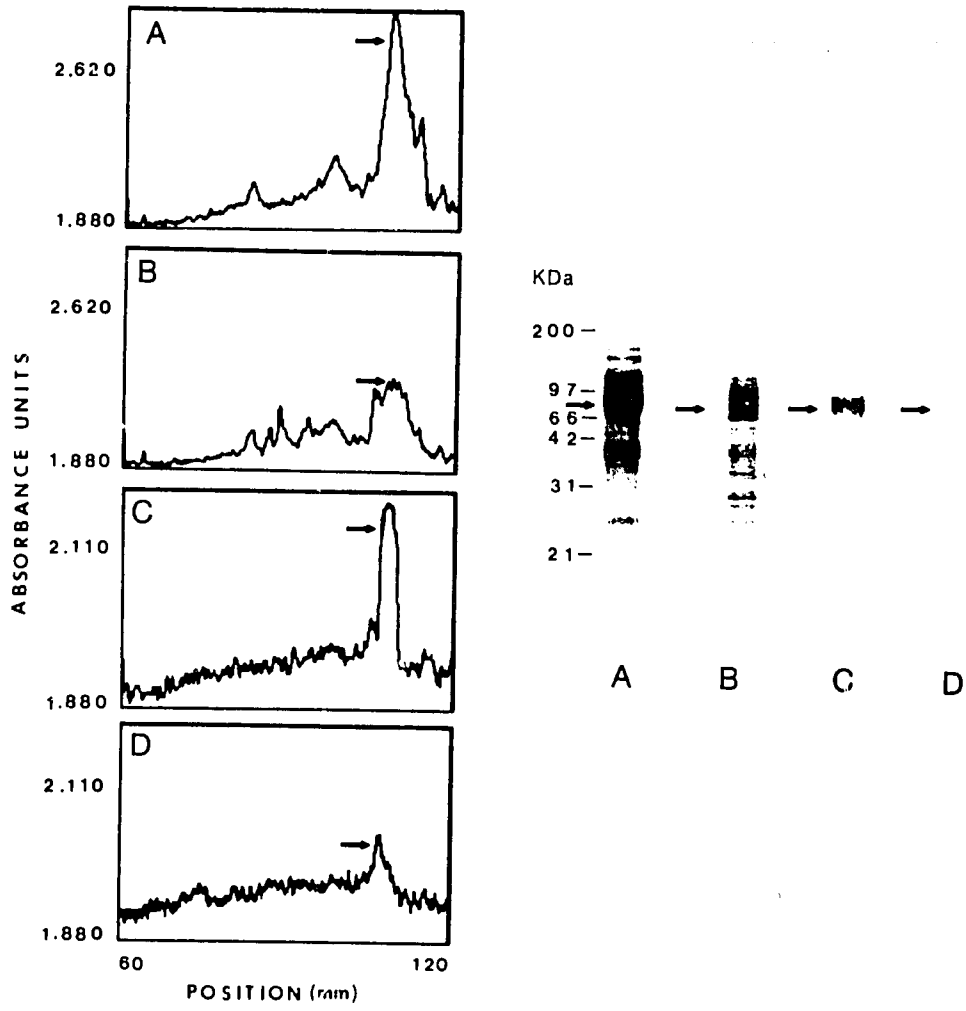

Fig. 4. Cell surface differences between highly and weakly infective trypomastigote clones and identification of the *T. cruzi* trypomastigote adhesion molecule that binds to myoblasts. Right panel (A, B): SDS-PAGE of biotinylated surface proteins of highly and weakly infective clones blotted onto nitrocellulose membranes and reacted with A-HRP as described in Materials and Methods. Right panel A, cell surface from highly infective clones. Right panel B, cell surface from weakly infective clones. Right panel (C, D): Molecular identification of the trypomastigote adhesion molecule that binds to rat heart myoblasts. Biotinylated proteins of trypomastigote clones were incubated with myoblasts. After removing unbound parasite material, the adhesion molecule was dissociated, transferred onto nitrocellulose membranes and reacted with A-HRP. Right panel C, 83 kDa from highly infective trypomastigotes that bound to myoblasts. Right panel D, 83 kDa from weakly infective trypomastigotes that bound to myoblasts. Left panel (A, B): Laser scan of biotinylated cell surface proteins from highly (A) and weakly (B) infected clones. Left panel (C, D): Laser scan of the biotinylated 83 kDa adhesion molecule from highly (C) and weakly (D) infective clones that binds to rat heart myoblasts. Arrows indicate the 83 kDa glycoprotein adhesion molecule. Blots were scanned from top to bottom. Molecular standards (BioRad) are indicated in kDa.

solubilized unlabeled trypomastigotes inhibited the binding of this molecule to heart myoblasts by 76%. The binding of solubilized radioiodinated trypomastigote clones MMC 20A and MMC 151D to heart myoblasts is concentration dependent and saturable as indicated in Fig. 5. Solubilized ^{125}I-labeled highly infective trypomastigotes from clone MMC 20A also bind more effectively to rat heart myoblasts than solubilized ^{125}I-labeled trypomastigotes from the weakly infective clone MMC 151D (Fig. 5). Autoradiograms obtained from SDS-PAGE of monolayers containing the bound parasite material in this assay indicate that the trypomastigote component mediating this

TABLE III

Effect of biotinylation of *T. cruzi* trypomastigote clones on their ability to attach to rat heart myoblasts

Parasite pretreatment	Parasite clone number	Percentage of cells containing attached parasites	Number of attached parasites per 100 cells
NHS-Biotin + DMF + PBS	20A	28.0 ± 2.4[a]	32.5 ± 3.7[a]
DMF + PBS	20A	25.0 ± 0.8	33.0 ± 2.3
PBS	20A	24.3 ± 1.2	35.3 ± 3.1
NHS-Biotin + DMF + PBS	151D	4.3 ± 0.2[a]	5.0 ± 0.2[a]
DMF + PBS	151D	4.5 ± 0.7	5.6 ± 1.6
PBS	151D	4.5 ± 1.8	4.8 ± 2.0

[a]The differences between test values and their controls were not statistically significant ($P < 0.05$).

binding is the 83 kDa and laser densitometry analysis of the autoradiograms indicate that the 83 kDa band from highly infective clones binds more pronouncedly to myoblasts than the 83 kDa band from the weakly infective clone (results not shown). SDS-PAGE of the dissociated parasite adhesion molecule under reducing or non-reducing conditions gives the same relative molecular mass of 83 kDa. In addition, SDS-PAGE gels of either CHAPS solubilized parasites or the dissociated parasite adhesion molecule stained with PAS and silver-Coomassie blue indicated that the 83 kDa molecule is a glycoprotein (data not shown).

Discussion

We have developed a model of highly and weakly infective *T. cruzi* trypomastigote clones that allowed us to identify a 83 kDa putative parasite binding molecule which mediates the attachment of trypomastigotes to myoblasts. Highly invasive trypomastigotes were found to express this glycoprotein in higher amounts and this could account for their marked increase in the ability to attach to myoblasts, when compared to weakly infective clones.

The biotinylated 83 kDa trypomastigote molecule specifically binds to the membrane of live rat heart myoblasts, since its binding is inhibited in a concentration dependent manner by solubilized unlabeled trypomastigotes in Western blots. This molecule appears to bind to heart myoblasts in a ligand receptor interaction manner since the specific binding is concentration dependent and saturable, as can be seen in Fig. 5. This binding is more effective in solubilized weakly infective trypomastigote clones (Fig. 5), and autoradiograms of SDS-PAGE from the dissociated bound trypomastigote material to myoblasts in these assays revealed only the 83 kDa band, more pronounced in samples from highly than weakly in-

TABLE IV

Inhibition of biotinylated trypomastigote 83 kDa binding to heart myoblasts by solubilized unlabeled trypomastigotes in Western blots, evaluated by laser scanning densitometry

Exp.	Excess of solubilized unlabeled trypomastigotes (mg protein)	Area of 83 kDa scanned (AU × mm)	% inhibition
1	0.00	0.43	0.0
	0.62	0.34	20.9
	1.25	0.26	39.5
	2.50	0.10	76.5
2	0.00	0.65	0.0
	0.62	0.54	16.2
	1.25	0.35	46.1
	2.50	0.20	69.2

These are two independent experiments of the same protocol carried out with the *T. cruzi* trypomastigote clone MMC 20A.

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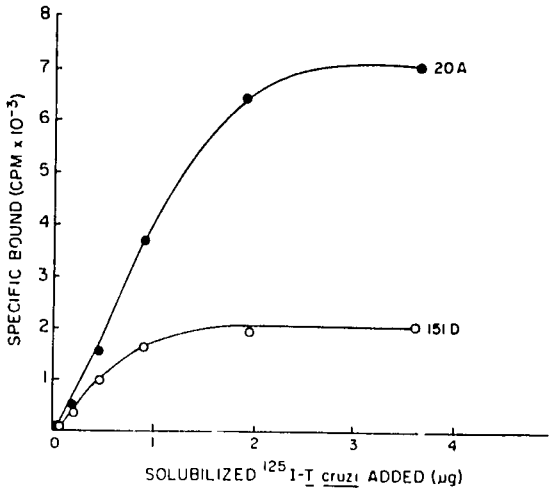

Fig. 5. Comparative binding of solubilized radioiodinated highly and weakly infective trypomastigote clones to myoblasts. Binding of solubilized radioiodinated highly invasive trypomastigote clone MMC 20A (●) and weakly invasive trypomastigote clone MMC 151D (○) to heart myoblasts. Increasing concentrations of solubilized ^{125}I-trypomastigote clones, starting with the same specific activity (1.27×10^5 cpm (µg protein)$^{-1}$) were incubated with full myoblast monolayers in 96-well plates for 4 h at 4°C. Specific binding was determined as described in Materials and Methods.

fective trypomastigote clones as shown by laser densitometry analysis (results not shown). These results indicate that the 83 kDa molecule, which is more abundant on the surface of highly than weakly invasive trypomastigote clones, mediates the interaction between trypomastigotes and heart myoblasts.

The highly and weakly infective clones that we have characterized are stable with regard to their properties of attachment, internalization, intracellular multiplication and membrane protein patterns. The stability of these properties is in agreement with results from other investigators who have found that clones derived from epimastigote forms of other strains of the parasite were genetically and antigenically stable over a period of years [19]. In addition, attachment and invasion did not change when clones were pre-incubated in fresh medium before being exposed to the host cell, suggesting that differences in these two parameters between these two groups of clones are not due to a maturation process of trypomastigotes as described by some investigators working with uncloned populations of *T. cruzi* [20]. It seems that *T. cruzi* strains are composed of heterogeneous populations differing in their attachment and invasiveness properties, since the Y strain of *T. cruzi* also presented clones that varied in their ability to infect myoblasts. The fact that *T. cruzi* trypomastigote populations are heterogeneous with respect to attachment and internalization into host cells, is in agreement with findings from other groups that showed that uncloned epimastigote forms of the parasite are heterogeneous with respect to growth kinetics [21], restriction endonuclease profiles [22], antigenic constitution [19,23,24] and that blood trypomastigote clones present differences in infectivity to mice [25].

Biotinylation or radioiodination of the surface proteins of trypomastigote clones did not affect the morphology, motility, parasite numbers and ability of the parasite to attach and invade myoblasts. Therefore, these processes would not modify the capacity of the trypomastigote clones to adhere to host cells. Analysis of the biotinylated or radioiodinated cell surface of these two types of clones revealed that they possessed differences in their protein profiles with respect to some components. Based upon these differences we identified a parasite 83 kDa glycoprotein, which was expressed more in highly infective than weakly infective clones, as the parasite ligand that mediates the attachment of parasites to myoblasts. Supporting this finding is the very recent observation suggesting that a molecule from uncloned populations of *T. cruzi* could mediate the interaction between the parasite and Vero cells [26] and previous work from another group also working with uncloned populations of *T. cruzi* that indirectly suggested the possible involvement of a *T. cruzi* 85 kDa molecule in this process [27].

Since this parasite adhesion molecule is a glycoprotein and sugar residues from cell surface glycoproteins on the parasite have been postulated to be involved in the interaction between the parasites and the host cells [3–8], we are investigating the possibility that the carbohydrate moiety of this molecule plays a role in the attachment of the parasite to the host cells.

This 83 kDa glycoprotein is recognized by antibodies produced against the cell surface of in-

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sect-derived trypomastigotes and blood trypomastigotes when analyzed by immunoblots (Lima, M.F., and Villalta, F., unpublished results). This observation indicates that this adhesion molecule may also be present on the surface of insect and blood derived trypomastigotes. Interestingly, antibodies produced during the course of a human infection, but not sera from uninfected individuals, also recognize this molecule by immunoblot (Lima, M.F., and Villalta, F., unpublished observations). These findings indicate that this 83 kDa adhesion molecule is immunogenic to man and could be used as candidate for human vaccination against Chagas' disease, if found to be protective.

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