

Involvement of the Stage-Specific 82-Kilodalton Adhesion Molecule of *Trypanosoma cruzi* Metacyclic Trypomastigotes in Host Cell Invasion

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This study provides several pieces of evidence indicating that 3F6-Ag, identified by monoclonal antibody (MAb) 3F6 as a stage-specific glycoprotein of approximately 82 kDa on the surface of metacyclic trypomastigotes of different *Trypanosoma cruzi* strains, promotes the entry of parasites into host cells through a ligand-receptor type interaction. First, invasion of Vero cells by metacyclic trypomastigotes of both CL and Tulahuen strains was significantly inhibited by MAb 3F6 or its Fab fragments. Second, purified 3F6-Ag bound to Vero cells in a dose-dependent and saturable fashion. Third, soluble 3F6-Ag reduced the infection of Vero cells by metacyclic forms of CL and Tulahuen strains by 90 to 97 and 50%, respectively. Unrelated proteins, as well as extracellular matrix components, such as heparan sulfate and collagen, had no effect. Our studies also show that in the Tulahuen strain, 10D8-Ag, a 35/50-kDa glycoprotein identified by MAb 10D8, participates in target cell invasion, confirming previous observations, but the variant form of 10D8-Ag expressed by highly invasive CL strain metacyclic trypomastigotes appears to be irrelevant. Overall, our results indicate that the surface components of *T. cruzi* metacyclic trypomastigotes involved in the process of host cell penetration are developmentally regulated molecules, such as 3F6-Ag and 10D8-Ag, that have no counterpart in blood- or tissue culture-derived trypomastigotes.

For many microorganisms that replicate intracellularly in the mammalian host, the crucial step for successful establishment of infection is the penetration into host cells, a process thought to be receptor mediated (6, 7, 10, 17). *Trypanosoma cruzi*, a protozoan parasite that causes Chagas' disease, can be included among such microbial agents.

Interaction of *T. cruzi* with host cells is initiated by metacyclic trypomastigotes, the developmental stages from insect vectors. Once inside the cells, the metacyclic forms escape from endocytic vacuoles to the cytoplasm and transform into amastigotes, which after several rounds of multiplication differentiate into trypomastigotes. Upon rupture of host cells, trypomastigotes circulate in the blood until they encounter appropriate target cells and then go through another intracellular cycle.

In the past 10 years, many laboratories have attempted to identify the surface components of *T. cruzi* implicated in host cell penetration. Several molecules of tissue culture-derived trypomastigotes, ranging from 80 to 250 kDa, have emerged as prospective ligands for target cell receptors, on the basis of either their cell binding capacity or the inhibitory effects of monoclonal antibodies (MAbs) (1, 3, 8, 21). Recently, Ortega-Barria and Pereira (16) have reported that penetrin, a novel *T. cruzi* heparin-binding protein of 60 kDa, promotes adhesion and penetration of trypomastigotes into mammalian cells. On the other hand, metacyclic trypomastigotes from insects or axenic cultures express a set of cell surface components that have no counterpart in blood or tissue culture trypomastigotes, such as the molecules of 90, 82, 75, 50, and 35 kDa (2, 24, 26), which may play a role in *T. cruzi*-host cell interaction. The doublet 35/50-kDa antigen

defined by MAb 10D8, for instance, appears to be enlisted for invasion of mammalian cells (20).

All of these findings point to the complexity of the cell invasion process by *T. cruzi*. Not only are metacyclic and blood trypomastigotes apparently equipped with distinct sets of molecules for target cell recognition and/or invasion, but it is also possible that different *T. cruzi* strains do not use the same repertoire of surface components to enter host cells. Furthermore, expression of variant forms of surface antigens has been observed among *T. cruzi* strains (14, 15), adding another variable to an already complicated picture.

In this study, we used metacyclic trypomastigotes of two different strains of *T. cruzi* to further investigate the surface molecules that promote parasite entry into mammalian cells. We examined in particular the role of 3F6-Ag, a stage-specific surface glycoprotein of approximately 82 kDa detectable by MAb 3F6 in different *T. cruzi* strains (24).

MATERIALS AND METHODS

Parasites. *T. cruzi* CL (4), Tulahuen (18), and G (25) were used. Parasites were maintained cyclically in mice and in liver infusion tryptose medium (5). To accelerate differentiation into trypomastigotes, *T. cruzi* was also grown in Grace's medium (GIBCO Laboratories, Grand Island, N.Y.) for 7 to 10 days before the parasites were harvested. Metacyclic trypomastigotes from Grace's medium or liver infusion tryptose cultures were purified through passage in a DEAE-cellulose column as previously described (24). In some experiments, *T. cruzi* cultures at the stationary growth phase, rich in metacyclic forms, were maintained for 24 h in the presence of tunicamycin (2 µg/ml), an inhibitor of N glycosylation of proteins.

Reagents. Type IV collagen was from Sigma Chemical Co.

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Radiolabeling of parasites, immunoprecipitation, and SDS-PAGE. The standard procedure for surface iodination (13) with ^{131}I , or metabolic labeling with L- ^{35}S methionine, of metacyclic trypomastigotes as well as procedures for preparation of parasite extracts, immunoprecipitation (11), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12) have been detailed elsewhere (24).

Purification of MAb 3F6. MAb 3F6 (24) was purified from ascitic fluid by using either a protein A-Sepharose (Pharmacia LKB, Uppsala, Sweden) or recombinant protein G-agarose (GIBCO BRL, Gaithersburg, Md.) column. Fab fragments were prepared by papain digestion and purification by protein A-Sepharose chromatography.

Purification of *T. cruzi* antigens. 3F6-Ag was purified from G strain metacyclic forms by passage in an antibody affinity column, prepared by coupling MAb 3F6 to CNBr-activated Sepharose 4B (Pharmacia LKB) as instructed by the manufacturer. Parasites were lysed with 0.5% Nonidet P-40 in phosphate-buffered saline (PBS) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), iodoacetamide (1 mM), antipain (25 $\mu\text{g}/\text{ml}$), and leupeptin (25 $\mu\text{g}/\text{ml}$). After centrifugation at $12,000 \times g$ for 5 min, the supernatant was collected and mixed with MAb 3F6-Sepharose for 2 h at 4°C under constant shaking. The resin was then packed at 3-cm height in a 10-ml plastic syringe, washed several times with PBS, and then washed three times with distilled water. The bound antigen was eluted with 50 mM triethylamine (pH 11.6) and dried in a speed vacuum concentrator. The amount of protein in the 3F6-Ag preparation was determined in 96-well microtiter plates by reaction with 0.01% (wt/vol) Coomassie brilliant blue G solution containing 5% ethanol and 10% (vol/vol) phosphoric acid, after which the optical density at 620 nm was read with a Multiscan MCC/340 P. The purity of the 3F6-Ag was evaluated by staining an SDS-polyacrylamide gel with Coomassie brilliant blue or by silver staining.

10D8-Ag was purified as previously described (19, 20). Briefly, preparations of 3×10^9 to 5×10^9 purified G strain metacyclic trypomastigotes were resuspended in 5 ml of distilled water, mixed with an equal volume of buffered phenol (pH 8.0), heated at 80°C for 15 min, and then centrifuged at $6,000 \times g$ for 20 min. The aqueous phase was collected, dialyzed against distilled water, and lyophilized. The dried preparation was resuspended in 400 μl of 10 mM Tris-HCl (pH 7.5) containing 10 mM MgCl_2 and treated with DNase and RNase (100 $\mu\text{g}/\text{ml}$) at 37°C for 3 h. The digested sample was extracted with phenol, dialyzed against distilled water, and dried in a speed vacuum concentrator. The amount of 10D8-Ag, which stained with Schiff reagent but not with Coomassie blue, was estimated by colorimetric method for sugar and related substances (9).

Cell cultures and parasite invasion assay. Growth of Vero cells and the trypomastigote invasion assay were carried out as already described (26). In standard invasion experiments, metacyclic trypomastigotes were incubated at 37°C for 3 h with Vero cells in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The parasite/cell ratio was 10:1 in all experiments. The rate of infection was determined by counting the number of intracellular parasites in 500 cells stained by Giemsa stain.

Binding of purified 3F6-Ag to Vero cells. For the enzyme-linked immunosorbent assay (ELISA), 10^5 Vero cells were seeded in 96-well vinyl microtiter plates (Costar, Cambridge, Mass.) and incubated overnight at 37°C . Upon fixation with

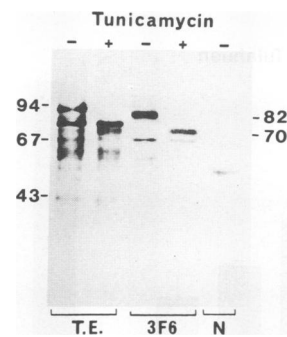


FIG. 1. SDS-PAGE analysis of *T. cruzi* 3F6-Ag. Metacyclic trypomastigotes (Tulahuen strain), nontreated or treated with tunicamycin (2 $\mu\text{g}/\text{ml}$), were metabolically labeled with L- ^{35}S methionine. After detergent lysis, total parasite extracts (T.E.), as well as the corresponding immunoprecipitates with MAb 3F6 or with normal mouse serum (N), were subjected to SDS-PAGE and fluorography. Note the shift in the electrophoretic migration of 3F6-Ag, from 82 to 70 kDa, after treatment with tunicamycin. Positions of molecular weight markers are shown on the left in kilodaltons.

4% paraformaldehyde in PBS, the cells were washed three times with PBS, blocked with PBS containing 10% FBS (FBS-PBS) for 1 h at 37°C , and incubated with purified *T. cruzi* antigen in FBS-PBS for 1 h at 37°C . After three washes with PBS containing 0.05% Tween 20 (T-PBS), the cells were incubated with MAb 3F6 at 20 $\mu\text{g}/\text{ml}$ for 30 min and then with anti-mouse immunoglobulin G conjugated to peroxidase for 30 min at 37°C . After washes with T-PBS, bound enzyme was revealed by adding *o*-phenylenediamine at 0.4 mg/ml in 10 mM Na_2HPO_4 -5 mM citric acid-0.06% H_2O_2 . After 10 min at room temperature in the dark, the reaction was stopped with 4 M H_2SO_4 and read in Multiscan MCC/340 P at 492 nm. To assay binding of 3F6-Ag to Vero cells, we also used the procedure described by Ortega-Barria and Pereira (16). Briefly, Vero cells grown almost to confluence in 24-well plates were rinsed once with 1% bovine serum albumin (BSA)-RPMI 1640 and incubated with increasing concentrations of ^{125}I -labeled 3F6-Ag at 37°C for 1 h. Thereafter, the medium was removed, Vero cells were washed three times with PBS and solubilized with 200 μl of 1 M NaOH, and bound radioactivity was measured in a gamma counter.

RESULTS

The *T. cruzi* antigen identified by MAb 3F6 (3F6-Ag). We have previously shown that MAb 3F6 reacts with an iodinated surface component of approximately 82 kDa in metacyclic forms of different *T. cruzi* strains (24). Here we show that 3F6-Ag is a glycoprotein containing N-linked oligosaccharide chains which migrates as a 70-kDa band (Fig. 1) after treatment of parasites with tunicamycin (2 $\mu\text{g}/\text{ml}$), an inhibitor of N glycosylation of proteins. MAb 3F6 also reacted with nonglycosylated *T. cruzi* recombinant protein expressed in *Escherichia coli* (data not shown), indicating that the peptide portion of 3F6-Ag, rather than its carbohydrate moiety, is the epitope recognized by MAb 3F6. 3F6-Ag is a developmentally regulated antigen that is not detectable in blood trypomastigotes, amastigotes, or epimastigotes (24). We have found that the gene encoding 3F6-Ag is transcribed predominantly in the metacyclic trypomastigote stage (data not shown).

Inhibition of in vitro *T. cruzi* infection by MAb 3F6. To test

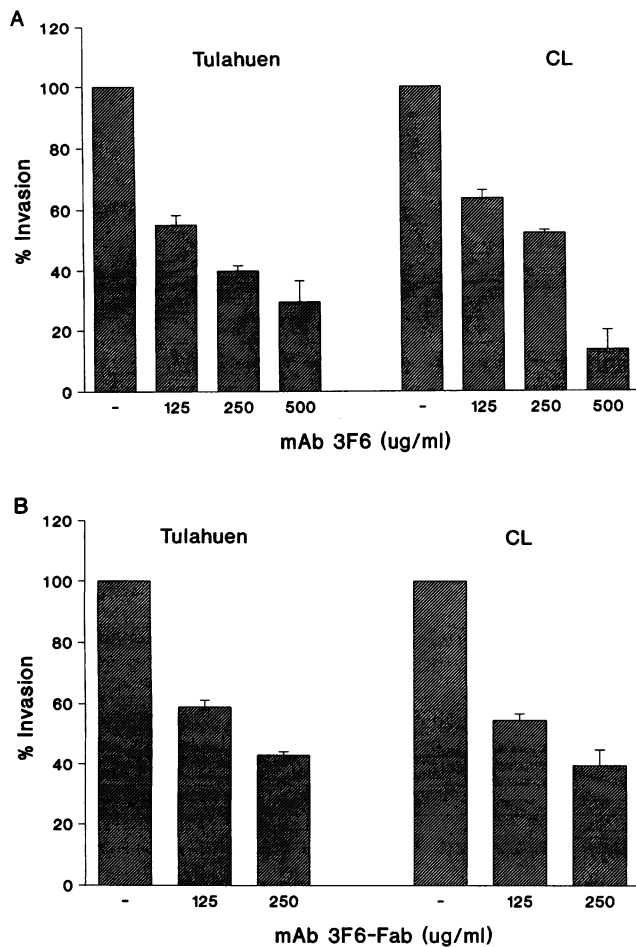


FIG. 2. Inhibitory effect of MAb 3F6 or its Fab fragments on *T. cruzi* entry into host cells. Metacyclic trypomastigotes of Tulahuen and CL strains were treated with the indicated concentrations of MAb 3F6 (A) or the corresponding Fab fragments (B) and then added to Vero cells. After 3 h at 37°C, the infection rate was determined by counting the number of intracellular parasites in a total of 500 Giemsa-stained cells. Values are means \pm standard deviations of two experiments performed in duplicate.

the ability of MAb 3F6 to block parasite entry into host cells, metacyclic trypomastigotes were incubated at room temperature for 30 min at the indicated concentrations of antibody and were then seeded onto Vero cells. After 3 h of incubation at 37°C, the rate of infection was determined by counting the number of intracellular parasites. As shown in Fig. 2A, infection of Vero cells was significantly reduced by MAb 3F6. Unrelated MAb 3D11, of the same isotype as MAb 3F6 (immunoglobulin G1) and directed to *Plasmodium berghei* sporozoites (27), had no effect. The inhibitory activity of MAb 3F6 was not due to a parasite-immobilizing, capping, or agglutinating effect, since the antibody does not exhibit any of these properties. More significant is that Fab fragments of MAb 3F6 also interfered with penetration of metacyclic forms into Vero cells (Fig. 2B), suggesting that MAb 3F6 could be affecting the interaction of 3F6-Ag with specific receptors on the Vero cell surface.

Binding of 3F6-Ag to Vero cell receptors. To determine the presence of receptors for 3F6-Ag in Vero cells, two binding assays were performed. In the ELISA, Vero cells immobi-

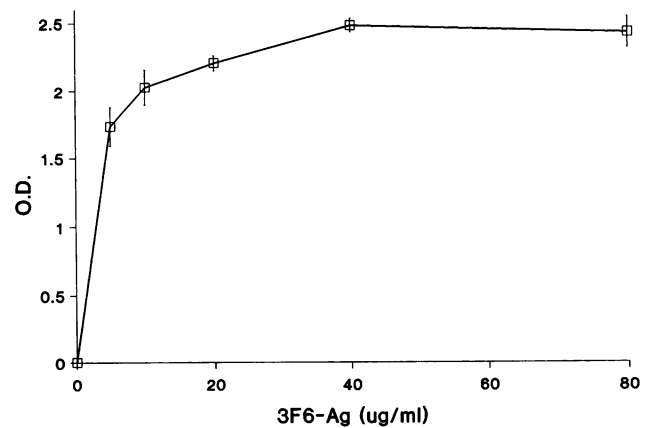


FIG. 3. Binding of 3F6-Ag to Vero cells. Increasing concentrations of purified 3F6-Ag were added to wells in ELISA plates containing Vero cells fixed with paraformaldehyde. After washes, the cells were sequentially incubated with MAb 3F6 and anti-mouse immunoglobulin conjugated to peroxidase. *o*-Phenylenediamine was used as the substrate to reveal the bound enzyme. Values are means of optical density (O.D.) at 492 nm \pm standard errors of triplicate experiments.

lized on the bottom of microtiter plates were incubated with increasing concentrations of purified 3F6-Ag. The antigen bound to Vero cells in a dose-dependent and saturable manner (Fig. 3). Essentially the same results were obtained in another set of experiments in which increasing concentrations of 125 I-labeled 3F6-Ag were incubated with live Vero cells (data not shown), and as expected, binding of labeled 3F6-Ag was blocked by nonlabeled homologous antigen (Fig. 4). Extracellular matrix (ECM) components, such as heparan sulfate and type IV collagen, that have been reported to negatively affect binding of *T. cruzi* penetrin to Vero cells (16) did not inhibit binding of 125 I-labeled 3F6-Ag (Fig. 4).

Effect of purified 3F6-Ag on host cell invasion by *T. cruzi*. If interaction of metacyclic trypomastigotes with target cells

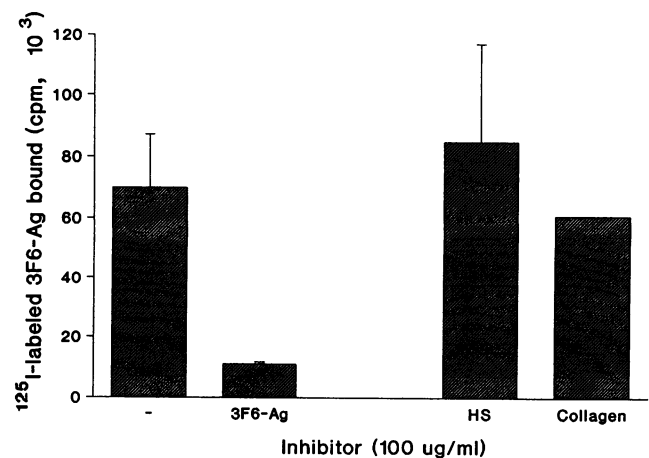


FIG. 4. Lack of effect of ECM components on the binding of 3F6-Ag to Vero cells. 125 I-labeled 3F6-Ag was preincubated with heparan sulfate (HS) or collagen in 1% BSA-RPMI and added to Vero cells. After washes, the radioactivity bound to Vero cells was measured. Values are means \pm standard deviations of duplicate experiments.

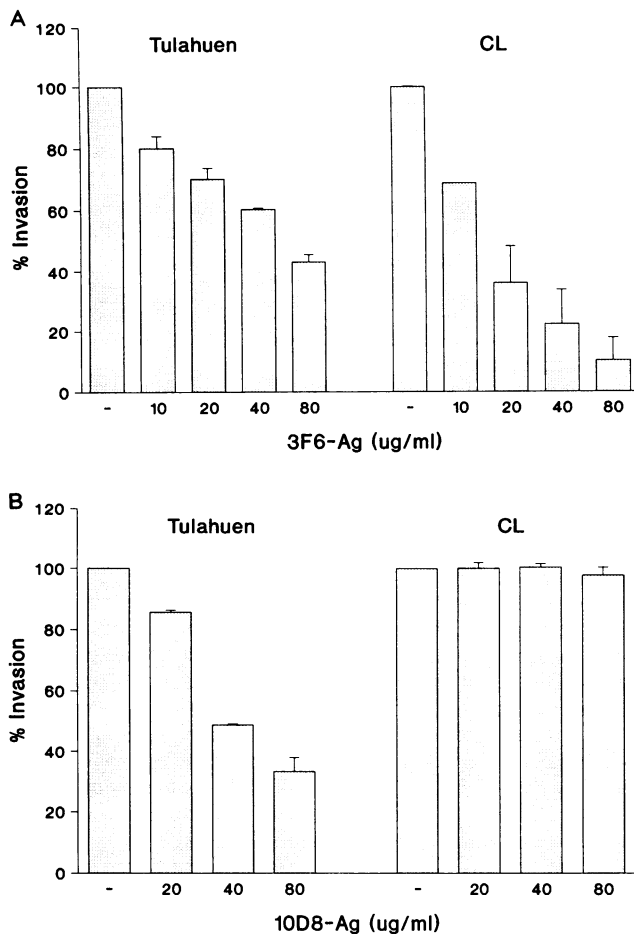


FIG. 5. Differential effect of purified *T. cruzi* antigens on penetration of host cells by parasites of different strains. Vero cells were incubated with purified 3F6-Ag (A) or 10D8-Ag (B) at the indicated concentrations before addition of metacyclic trypomastigotes. After 3 h at 37°C, the number of intracellular parasites was counted in a total of 500 Giemsa-stained cells. Values, expressed as percentage of invasion, are means \pm standard deviations of two experiments performed in duplicate.

was in fact mediated by 3F6-Ag, the soluble antigen would be able to affect parasite invasion. This possibility was investigated by incubating Vero cells with various concentrations of affinity-purified 3F6-Ag at 37°C for 10 min and then adding metacyclic trypomastigotes of the CL or Tulahuen strain. Upon incubation at 37°C for 3 h, the number of intracellular parasites was counted. Infection of Vero cells was inhibited by 3F6-Ag in a dose-dependent manner (Fig. 5A). Irrelevant proteins, as well as heparan sulfate and type IV collagen, had no effect (data not shown). The inhibitory effect of 3F6-Ag was always higher for the CL strain than for the Tulahuen strain. At 80 μ g/ml, 3F6-Ag reduced the entry of CL strain metacyclic trypomastigotes into Vero cells by more than 90% and that of Tulahuen strain metacyclic trypomastigotes by 50% (Fig. 5A).

Invasion of Vero cells by mammalian cell-derived trypomastigotes was not inhibited by purified 3F6-Ag, which is consistent with the lack of expression of 3F6-Ag by these developmental stages.

Differential effect of 10D8-Ag on Vero cell invasion by metacyclic forms of different *T. cruzi* strains. The soluble

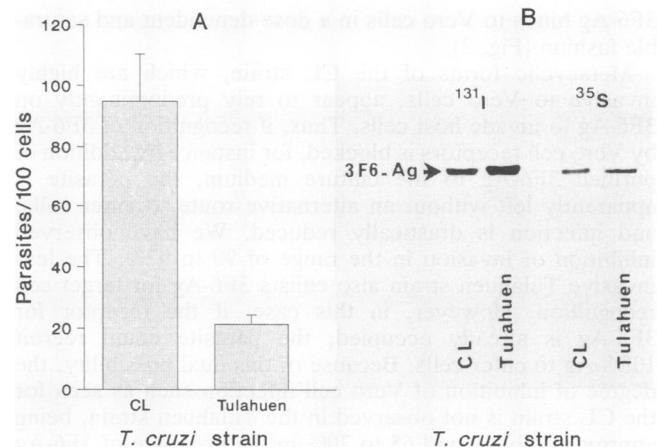


FIG. 6. Differential infective capacity of metacyclic trypomastigotes of different *T. cruzi* strains and lack of association with degree of expression of 3F6-Ag. (A) Metacyclic forms of CL and Tulahuen strains were incubated with Vero cells for 3 h at 37°C, and then the number of intracellular parasites was counted. Values are means \pm standard errors of five independent experiments. (B) SDS-PAGE profile of metacyclic trypomastigotes labeled with ¹³¹I or with [³⁵S]methionine and immunoprecipitated with MAb 3F6.

form of 10D8-Ag, another metacyclic trypomastigote-derived antigen, was unable to inhibit infection of Vero cells by CL strain metacyclic forms but significantly reduced Vero cell infection by Tulahuen strain metacyclic forms (Fig. 5B). Since CL strain metacyclic trypomastigotes bear a 10D8-Ag which is a variant form of the molecule expressed by the Tulahuen strain (15), it is possible that the CL strain 10D8-Ag is not even recognized by target cell receptors.

Differential cell invasion capacity of CL and Tulahuen strain metacyclic trypomastigotes. Throughout our studies, we have observed a higher rate of infection of host cells by the CL strain than by the Tulahuen strain. Figure 6A shows that the average number of CL strain metacyclic forms that enter Vero cells is at least four times greater than the number of Tulahuen strain metacyclic forms. This difference does not appear to be associated with the degree of expression of 3F6-Ag, since the intensity of the 3F6-Ag band was higher in Tulahuen than in CL strain metacyclic forms, either in fluorographs of [³⁵S]methionine-labeled immunoprecipitates or in radioautograms of iodinated samples (Fig. 6B).

DISCUSSION

One of the common features of *T. cruzi* metacyclic trypomastigotes, the developmental stages that initiate infection, and blood trypomastigotes, which propagate infection within the mammalian host, is the ability to invade host cells. However, these two types of trypomastigotes appear to mobilize different surface molecules for target cell invasion. Our results strongly support this assumption.

We have found that infection of Vero cells by CL or Tulahuen strain metacyclic trypomastigotes can be significantly inhibited either by MAb 3F6 or its Fab fragments (Fig. 2) or by purified 3F6-Ag (Fig. 5) in a dose-dependent manner. These data indicate that the stage-specific 3F6-Ag, which is expressed on the surface of metacyclic forms of different *T. cruzi* strains (24), is implicated in the process of parasite entry into host cells. We envisage that cell invasion is initiated upon interaction of 3F6-Ag with specific Vero cell receptors, which is compatible with the observation that

3F6-Ag binds to Vero cells in a dose-dependent and saturable fashion (Fig. 3).

Metacyclic forms of the CL strain, which are highly invasive to Vero cells, appear to rely predominantly on 3F6-Ag to invade host cells. Thus, if recognition of 3F6-Ag by Vero cell receptors is blocked, for instance by addition of purified 3F6-Ag to the culture medium, the parasite is apparently left without an alternative route to enter cells, and infection is drastically reduced. We have observed inhibition of invasion in the range of 90 to 97%. The less invasive Tulahuen strain also enlists 3F6-Ag for target cell recognition. However, in this case, if the receptor for 3F6-Ag is already occupied, the parasite could recruit 10D8-Ag to enter cells. Because of this dual possibility, the degree of inhibition of Vero cell infection such as seen for the CL strain is not observed in the Tulahuen strain, being approximately 50 and 65 to 70% in the presence of 3F6-Ag and 10D8-Ag, respectively (Fig. 5).

At present, we do not know whether the role of 3F6-Ag in host cell invasion is as critical in other highly infective *T. cruzi* strains as it appears to be in CL strain metacyclic forms. Another question that remains unanswered is why CL strain metacyclic trypomastigotes enter target cells in such a high number compared with the Tulahuen strain. One simple explanation is that expression of 3F6-Ag is greater in CL strain metacyclic forms. However, this view is not supported by experimental data indicating that the CL strain expresses comparable or even lower amounts of 3F6-Ag on the surface (Fig. 6). An interesting possibility is that the lack of recognition by host cells of CL strain 10D8-Ag, which has been shown to be a variant form of the molecule present in the Tulahuen and G strains (15), would facilitate invasion. In this case, expression of the correct 10D8-Ag, which binds to its counterreceptors on target cells, would be disadvantageous. Our speculative view is that the route of parasite entry mediated by receptors for 3F6-Ag is much more efficient than that dependent on receptors for 10D8-Ag. In the Tulahuen strain, commitment of 10D8-Ag to cell invasion, through a less efficient pathway, would hamper the use of 3F6-Ag and consequently the entry of parasites. In this regard, it is suggestive that G strain metacyclic forms, which bear 10D8-Ag of the same type as that in the Tulahuen strain and in larger amounts (15), is even less invasive, the average infection rate being limited to about 10%, as opposed to 20 and 100% for Tulahuen and CL strains, respectively.

Which portion of 3F6-Ag or 10D8-Ag makes the contact with receptor molecules remains to be established. Since both 3F6-Ag and 10D8-Ag are carbohydrate-containing molecules, the sugar moiety could potentially participate in the interaction with receptors. 10D8-Ag is a mucin-like molecule; i.e., it is a highly glycosylated protein with high serine and threonine composition bearing O-linked glycans (22). In analogy to a mucin-like molecule of mammalian cells identified as part of the ligand for lymphocyte homing receptors, L-selectin (23), interaction of 10D8-Ag with its receptors may be of the lectin-carbohydrate type. At least, this possibility is compatible with the observation that the epitope recognized by MAb 10D8, which inhibits parasite penetration, is a carbohydrate (26). On the other hand, we have observed that MAb 3F6, which inhibits *T. cruzi* infection, reacts with 3F6-Ag devoid of its N-linked oligosaccharides and also with carbohydrate-free recombinant protein, pointing to the possibility that the peptide portion of the molecule associates with the host cell receptor.

No information about the nature of 3F6-Ag or 10D8-Ag receptors is available. Ortega-Barria and Pereira (16) have

suggested that heparin- or collagen-like materials could be the receptors for *T. cruzi* penetrin, a protein that promotes infection and has the ability to bind ECM components. These authors have observed that collagen, heparin, and heparan sulfate, in particular the latter, inhibit the penetration of tissue culture-derived trypomastigotes into Vero cells, by blocking association of penetrin with target cell receptors. We have examined the ability of metacyclic trypomastigotes to bind collagen or heparan sulfate, with negative results. Accordingly, these ECM components were unable to inhibit penetration of CL or Tulahuen metacyclic trypomastigotes into Vero cells. Receptors for 3F6-Ag or 10D8-Ag are therefore unlikely to be collagen-like or heparin-like molecules.

One point that should be emphasized is that 3F6-Ag and 10D8-Ag are both developmentally regulated molecules, expressed by metacyclic trypomastigotes from axenic cultures or from insect vectors but undetectable in blood trypomastigotes (24). All of these data, together with the findings on differential association of metacyclic and tissue culture-derived trypomastigotes with mammalian ECM elements, strongly suggest that the two types of trypomastigotes evolved distinct means of associating with host cells and host components. This is not surprising when one takes in consideration the natural habitat of these developmental stages. Blood trypomastigotes, during their long residence within the mammalian host, encounter widely diverse types of cells and humoral factors in addition to ECM material that they must traverse. In contrast, contact of metacyclic trypomastigotes with mammalian host cells is transient. Upon entering the host organism, metacyclic forms could immediately invade cells at the very site of parasite entry, potentially before any extensive interaction with surrounding components.

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