This research grant was designed to define the role of a host iron-binding protein on Trypanosoma cruzi multiplication and development. The ultimate significance of this work is that an understanding of the basic mechanisms by which T. cruzi, a human parasite that affects millions of people in South and Central America, takes up micronutrients from the host may facilitate the formulation of rational strategies for blocking the multiplication of the intracellular forms of this parasite in the mammalian host.

The specific objectives of this grant were: (a) to determine the specificity and number of receptors for human transferrin, an iron-binding protein, on amastigote and trypomastigote forms of T. cruzi, (b) to study the involvement of human transferrin on the transport of iron to T. cruzi,
(c) to study the effect of iron on \textit{T. cruzi} amastigote multiplication, and (d) to identify the parasite receptor for transferrin and compare its properties with the host cell receptor for transferrin.

We have successfully achieved these four goals in this grant. In a brief summary, the results obtained show that \textit{T. cruzi} amastigotes present receptors for human transferrin. However, \textit{T. cruzi} trypomastigotes do not bind to human transferrin. We found that in the presence of physiological concentrations of ferrotransferrin, this ligand binds to its receptors on the amastigote form, which is a 200 kilodalton protein. Our results show that iron, which we found to be essential for \textit{T. cruzi} amastigote growth, is delivered from ferrotransferrin to amastigotes by receptor mediated endocytosis. It is interesting that unlike mammalian cells, which present only one type of receptor for transferrin with a $K_d$ of $10^{-12}$M and apparent molecular weight of 180 kilodaltons, \textit{T. cruzi} amastigotes present two classes of receptors with a $K_d$ of $10^{-12}$M and an apparent molecular weight of 200 kilodaltons. These differences between the host cell and \textit{T. cruzi} amastigote receptors for transferrin suggest that this human parasite may present a peculiar mechanism by which iron is taken from the host and it could offer a novel approach to blocking the infection.

**Summary of research findings**

\textit{Trypanosoma cruzi} amastigotes present receptors for human transferrin as indicated by the saturable binding of $^{125}$I-transferrin to this form of the parasite. Scatchard analysis revealed two classes of receptors present at $4 \times 10^4$ and $7.1 \times 10^4$ receptors per amastigote with a $K_d$ of $1.1$ and $4.3 \times 10^{-12}$M respectively. In contrast, trypomastigotes did not bind human transferrin. Iron is required for amastigote growth in cell free-medium since deferoxamine, an iron chelator, inhibits amastigote growth and which is restored when deferoxamine is removed from the medium. $^{59}$Fe-transferrin which bound to amastigotes at $4^\circ$C for 1 hour was readily dissociated from the parasite surface upon treatment with acid. However, this treatment did not disrupt the binding that occurred at $37^\circ$C for 1 hour. Amastigote growth in cell-free medium is inhibited in ferrotransferrin-depleted serum and addition of ferrotransferrin restores parasite growth. Immunoblots of solubilized amastigote membranes probed with anti-human transferrin receptor indicate that a protein of molecular weight 200 kDa interacts with transferrin. Western blots of bioxidated amastigote surface proteins demonstrate that this protein is present on the cell surface, therefore human transferrin seems to interact with a 200 kDa surface amastigote receptor. Iron, which is essential for amastigote growth is delivered to \textit{T. cruzi} amastigotes by receptor mediated endocytosis through these receptors. A full manuscript containing these results was submitted for publication to \textit{Molecular and Biochemical Parasitology}: Lima, M.F. and Villalta, F. \textit{Trypanosoma cruzi} receptors for human transferrin and its role.

In addition to the results presented above, we were able to study the binding of another human protein, fibronectin, to \textit{T. cruzi} amastigotes. We have investigated the role of this human protein on \textit{Trypanosoma cruzi} amastigote binding to and uptake by murine macrophages and human monocytes.

In a brief summary, we found that \textit{T. cruzi} amastigotes present receptors for human fibronectin. Our results indicate that in the presence of physiological concentrations of fibronectin, these receptors increase the binding to and uptake of amastigotes by both murine macrophages and human monocytes. These studies elucidate the mechanism by which \textit{T. cruzi} amastigotes are recognized by macrophages and monocytes. Macrophage-monocyte interactions with \textit{T. cruzi} amastigotes are important because these phagocytic cells play a role in the immunomodulation of the host response to \textit{T. cruzi}. A better understanding of these processes would facilitate an appropriate immunological manipulation in favor to the host against \textit{T. cruzi} infection.

**Summary of research findings**

\textit{T. cruzi} amastigotes present receptors for human fibronectin as indicated by the saturable binding of $^{125}$I-fibronectin to this form of the parasite. Scatchard analysis indicates that the number of fibronectin receptors per amastigote is $1.3 \times 10^5$ with a $K_d$ of approximately $2.3$ nM. Addition of physiological concentrations of fibronectin to amastigote-macrophage co-cultures significantly increases the binding of amastigotes to murine macrophages. This increase is
evidenced in both the number of amastigotes bound to macrophages and the percentage of macrophages containing bound amastigotes. The uptake of amastigotes by either murine macrophages or human blood monocytes is also increased in the presence of exogenous fibronectin. The increase induced by fibronectin is blocked when amastigotes are pretreated with the RGDS tetrapeptide of the fibronectin cell attachment site. Furthermore, the ability of fibronectin to enhance amastigote binding to and uptake by macrophages is inhibited by the F(ab')₂ fragment of anti-fibronectin immunoglobulin G (IgG) but not by an irrelevant anti-human IgG F(ab')₂ fragment. Pretreatment of either amastigote or macrophages with fibronectin also results in a significant increase in amastigote binding to and uptake by macrophages. These results suggest that fibronectin plays a role in facilitating the attachment and ingestion of T. cruzi amastigotes by macrophages and monocytes in chagasic tissue lesions. These results were accepted for publication as a full paper: Noisin, E.L. and Villalta, F. 1989. Fibronectin increases Trypanosoma cruzi amastigote binding to and uptake by murine macrophages and human monocytes. Infection and Immunity, in press (April issue).

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Haiti (no cost to the project)
Trypanosoma cruzi RECEPTORS FOR HUMAN TRANSFERRIN
AND ITS ROLE

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Abbreviations: A-AP, Avidin alkaline phosphatase; CHAPS, (3-[(3-(chlolamidopropyl) dimethyl-ammonio] 1-propanesulfonate); HBSS, Hanks’ Balanced Salt solution, pH 7.2; PBS, phosphate buffered saline, pH 7.2; PMSF, phenyl-methylsulfonylfluoride; NHS-biotin, N-hydroxisuccinimide biotin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLCK, N-p-tosyl-L-Lysine-chloro-methyl ketone.
(Summary)

*Trypanosoma cruzi* amastigotes present receptors for human transferrin as indicated by the saturable binding of $^{125}$I-transferrin to this form of the parasite. Scatchard analysis revealed two classes of receptors present at $4 \times 10^4$ and $7.1 \times 10^4$ receptors per amastigote with a $K_d$ of 1.1 and $4.3 \times 10^{-12} M$ respectively. In contrast, trypomastigotes did not bind human transferrin. Iron is required for amastigote growth in cell free-medium since deferoxamine, an iron chelator, inhibits amastigote growth and growth is restored when deferoxamine is removed from the medium. $^{59}$Fe-transferrin which bound to amastigotes at $40^\circ C$ for 1h was readily dissociated from the parasite surface upon treatment with acid. However, this treatment did not disrupt binding that occurred at $37^\circ C$ for 1h. Amastigote growth in cell-free medium is inhibited in ferrotransferrin-depleted serum and addition of ferrotransferrin restores parasite growth. Western blots of solubilized amastigote membranes probed with anti-human transferrin receptor indicate that a protein of molecular weight 200 kDa interacts with transferrin. Western blots of biotinylated amastigote surface proteins demonstrate that this protein is present on the cell surface, therefore, human transferrin seems to interact with a 200 kDa surface amastigote protein receptor. Iron, which is essential for amastigote growth, could be delivered to *T. cruzi* amastigotes by receptor mediated endocytosis through these receptors.
Key words: *Trypanosoma cruzi*, amastigote receptors for human transferrin, iron delivery from ferrotransferrin, receptor mediated endocytosis, ferrotransferrin dependent amastigote growth.
INTRODUCTION

Trypanosoma cruzi, a protozoan affecting millions of people in South and Central America, requires an intracellular location to multiply and amplify the disease in mammalian hosts [1]. T. cruzi has a complex life cycle and is found in the mammalian host both as the intracellular amastigote and the extracellular non-dividing, but host-cell invasive, bloodstream trypomastigote forms. The amastigote form is the multiplicative stage in the mammalian host and transforms into the trypomastigote form intracellularly.

The basic mechanisms whereby T. cruzi amastigotes take up micronutrients from the host to multiply in mammalian cells are largely unknown. An understanding of these basic mechanisms may facilitate the formulation of rational strategies for blocking the multiplication of the intracellular forms of this human parasite in the mammalian phase of the cycle.

We have been investigating the involvement of host proteins in the transport of micronutrients to amastigotes. Transferrin is a major iron transport protein of mammalian cells [2] and transferrin receptors have been found in virtually all cells and in increased amounts during cell proliferation [3-5]. Since amastigotes are the multiplicative form of the parasite in the
mammalian host we decided to investigate in this work the binding of human transferrin to T. cruzi amastigotes and the consequences of this interaction.

**MATERIAL AND METHODS**

**Parasites.** The Tulahuen strain of T. cruzi was used in this work. Blood trypomastigotes isolated from mice by chromatography on a diethylaminoethyl-cellulose column [6] were used to infect Vero cell cultures [7]. Amastigotes released from these infected cells were isolated on a metrizamide gradient [7] and grown in modified ML-15HA medium [7] without hemin. Parasites were washed by centrifugation with Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) and resuspended at the appropriate concentrations in media as described below.

**Radioiodination of transferrin.** Purified human ferrotransferrin was obtained from Sigma Chemical Company (St. Louis, MO). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis [8] of this protein revealed a sharp band of 81 kilodaltons. Transferrin (1mg) was mixed with 1 mCi of $^{125}$I (specific activity, 17 mg$^{-1}$ I; ICN Biochemicals, Irvine, CA) in Iodogen (Pierce Chemical Co, Rockford, IL)-coated tubes [9] at room temperature for 15 min. Unbound radioactivity was removed by
gel filtration through Sephadex G-25 (Pharmacia, Inc. Piscataway, N.J.). Radiolabeled transferrin was concentrated by ultrafiltration in Centrisart I tubes (Vangard International, Inc. Neptune, N.J.). The specific activity of the concentrated $^{125}\text{I}$-labeled transferrin was $2.1 \times 10^6$ to $5.5 \times 10^6$ cpm (ug protein)$^{-1}$.

**Labeling of apotransferrin with \(^{59}\text{FeCl}_3\).** Purified human apotransferrin (Sigma) was labeled with \(^{59}\text{FeCl}_3\) by the nitriloacetic acid method as described [10]. Briefly apotransferrin (8 mg) was dissolved in 0.25M Tris-Cl buffer in the presence of 0.1M NaHCO$_3$. This solution was mixed with 100 mM disodium nitriloacetate and 0.125 mCi \(^{59}\text{FeCl}_3\) (specific activity 29.3 mCi mg$^{-1}$ Fe at 0.341 mg Fe ml$^{-1}$; ICN Biochemicals) for 1 h at room temperature. Unbound radioactivity was removed by gel filtration through Sephadex G-25. \(^{59}\text{Fe}\)-labeled transferrin was concentrated by ultrafiltration as described above. Transferrin preparations were routinely found to be fully saturated since the ratio $A_{280}/A_{450}$ was 0.1 which is consistent with full saturation [11].

**Binding assays.** The binding of $^{125}\text{I}$-transferrin to amastigotes was carried out by adding to Eppendorf tubes (1.5 ml), precoated with 20% BSA, 50-ml portions of the amastigote suspensions (4 x $10^6$ amastigotes ml$^{-1}$ in Hanks' Balanced Salt
solution pH 7.2 supplemented with 1% BSA, HBSS-BSA) followed by 50 ul of HBSS-BSA containing increasing concentrations of $^{125}$I labeled transferrin (50 to 600 ug ml$^{-1}$ in HBSS-BSA) and 50 ul of HBSS-BSA alone or containing 100-fold excess unlabeled transferrin to determine nonspecific binding. Each point was done in triplicate. The tubes were incubated at room temperature with constant shaking for 1 h. Unbound radiolabeled transferrin was removed by centrifugation at 4°C. Specific binding was determined by subtracting nonspecific binding (determined in the presence of excess unlabeled transferrin) from the total amount of counts.

In addition, binding of human transferrin to the surface of the parasite was evaluated by indirect immunofluorescence [12,13]. Amastigotes or culture trypomastigotes obtained from myoblasts [14,15] were fixed with 0.25% formaldehyde and incubated (37°C, 30 min) with PBS + BSA alone or containing 1000 to 2000 ug transferrin ml$^{-1}$. After several washings, these preparations were incubated (37°C, 30 min) with heat-inactivated normal rabbit serum, washed, and then incubated (37°C, 30 min) with a solution of goat anti-transferrin IgG. After several washings the slides were incubated (37°C, 30 min) with fluorescein labeled rabbit anti-goat IgG (Cappel, West Chester, PA). The slides were then washed, air dried and examined with a fluorescence microscope.
Binding to and dissociation of $^{59}$Fe-transferrin from amastigotes. Triplicate groups of Eppendorf tubes (1.5 ml) received 50-μl portions of the amastigote suspension (2 x $10^7$ amastigotes ml$^{-1}$ in HBSS) followed by 50 μl of HBSS containing 400 μg ml$^{-1}$ of $^{59}$Fe-labeled transferrin or HBSS alone. Unbound $^{59}$Fe-transferrin was removed by ultrafiltration at 4°C. Amastigotes were then treated with 50 μl of 0.25M acetic acid in 0.5M NaCl for 5 seconds at 4°C. These conditions were found to dissociate bound transferrin from its receptor on mammalian cells [11]. The pH of the cultures was immediately neutralized to 7.0 by the addition of 1M sodium acetate. Cells were centrifuged in an Eppendorf centrifuge at 13,000 x g for 5 min and radioactivity associated with the pellets was determined in a gamma counter. This short treatment of amastigotes with acetic acid did not affect the morphology of the parasite, the amastigote concentration in the pellet or their ability to grow in ML-15HA.

Biotinylation of amastigotes. Amastigote cell surface proteins were biotinylated by adding a 2.6 mM final concentration of N-hydroxysuccinimide biotin (NHS-Biotin) (Bio-Rad, Richmond, CA), dissolved in dimethyl formamide, to 5 x $10^8$ parasites resuspended in PBS for 15 min at room temperature [14,15]. Amastigotes retained similar morphology 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)-dimethylamonio] 1-propane-sulfonate (CHAPS) [14,15] in PBS in the presence of protease inhibitors [1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM p-tosyl-L-lysinechloromethylketone (TLCK), and 2.8 ug ml⁻¹ aprotinin]. Solubilized amastigotes were centrifuged at 13,000 x g at 4°C for 5 min to remove debris.

**Western blots.** Ten ug of biotinylated parasite samples obtained as described above were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% acrylamide gel, in the presence or absence of B-mercaptoethanol according to Laemmli [8] in a mini PROTEAN II cell (Bio-Rad). Gels were then electroblotted onto 0.45 um nitrocellulose membranes (Bio-Rad) for 1 h at 4°C in a mini Trans Blot cell (Bio-Rad). Biotinylated molecular weight markers (Bio-Rad) were included in the gels to determine the relative molecular weight of parasite samples. Nitrocellulose membranes were then stained with avidin-alkaline phosphatase (A-AP; Bio-Rad), and developed.

**Immunoblots.** The molecular weight of the amastigote receptors for human transferrin were identified by immunoblots. Membrane enriched preparations were prepared as described in detail [16], subjected to SDS-PAGE and blotted onto nitrocellulose membranes as described above. The nitrocellulose strips were then reacted
with a 5% solution of non fat dry milk in PBS for one hr at room
temperature, and incubated with a 1:50 dilution of the monoclonal
B3/25 specific for human transferrin receptor (Boehringer-
Manheim, CA) in 5% solution of nonfat dry milk overnight at
4°C. Membranes were then washed with Tris buffered saline
solution pH 7.5 containing 0.2% Tween (TTBS) and incubated with a
1:100 dilution of goat anti mouse IgG labeled with alkaline
phosphatase (Bio-Rad) for 1 h at room temperature. Membranes were
washed two times for 5 min with TTBS, two times for 5 min with
Tris buffered saline pH 7.5 (TBS) and developed.

Amastigote growth in the presence and absence of
deferoxamine. Triplicate aliquots of amastigote suspensions were
incubated in flat-bottom microculture wells (Limbro, New Haven,
CT) containing 0.1 ml portions of amastigote suspension (2 x 10^7
organisms ml\(^{-1}\) in ML-15HA medium), 0.01 ml of deferoxamine
solution (Desferal mesylate, Ciba-Geigy, Summit, NJ) at 2.5 mg
ml\(^{-1}\) in ML-15HA and 0.79 ml of ML-15HA medium. Control cultures
received 0.01 ml of ML-15HA medium instead of deferoxamine.
Amastigote cultures were incubated at 37°C in a 5% CO\(_2\)-in-air-
incubator saturated with water vapor as described [7]. The
concentration of amastigotes was determined microscopically with
a Neubauer hemocytometer at 24 h intervals. At the end of the
experiments, deferoxamine was removed from amastigote cultures by
washing the cells three times with HBSS and resuspending the
parasites in fresh ML-15HA medium. These cultures were incubated in the same conditions as described above and amastigote growth was determined microscopically.

Amastigote growth in medium supplemented with human serum depleted of transferrin and supplemented with ferrotransferrin. In these experiments heat inactivated serum from healthy volunteers was used to supplement ML-15HA medium [7]. Human serum was depleted of transferrin by passing through an affinity chromatography Affi-Gel H\(\text{z}\) column (Bio-Rad) coupled to goat anti-human transferrin IgG. The coupling of anti-human IgG (3 mg ml\(^{-1}\)) to Affi-Gel H\(\text{z}\) was performed as described by the manufacturer. SDS-PAGE of the run-off material stained with silver did not show the 81 kDa band corresponding to human transferrin. In addition, no band was observed when the eluted material from the column was incubated with goat anti-human transferrin IgG by immunodiffusion. Control human serum was treated similarly but eluted from a column containing an irrelevant goat anti-human IgG coupled to Affi-gel H\(\text{z}\). In contrast, human serum chromatographed under these conditions presented the typical 81 kDa band of transferrin.

Ferrotransferrin (100% Fe-saturated human transferrin) was purchased from Sigma. ML-15HA medium [7] does not contain ferrotransferrin, apotransferrin or any source of iron.

Amastigote cultures were washed three times with HBSS and triplicate aliquots of parasite suspensions were incubated in
plastic microcultures plates (Limbro) containing 0.02 ml of amastigote suspension (1.55 x 10^8 organisms ml^{-1} in ML-15HA medium), 0.9 ml of ML-15HA supplemented with 10% human serum depleted of transferrin as described above, 0.9 ml of ML-15HA supplemented with 10% human serum, 0.9 ml of ML-15HA supplemented with 3 mg ml^{-1} of ferrotransferrin or apotransferrin and 10% transferrin-depleted human serum. The cultures were incubated as described above and amastigote growth was evaluated in a hemocytometer [7]

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

**RESULTS**

**Binding of labeled transferrin to amastigotes.** The binding of 125I-transferrin to *T. cruzi* amastigotes was specific and saturable, indicating the presence of transferrin receptors in this form of the parasite (Fig. 1). As indicated in the insert of Fig. 1, Scatchard analysis [17] of the binding data indicates that there are two classes of receptors for transferrin present.
at $4.0 \times 10^4$ and $7.0 \times 10^4$ per amastigote with a Kd of 1.1 and $4.3 \times 10^{-12}$M respectively. Pre-incubation of amastigotes with excess (100-fold) cold transferrin for 5 min resulted in a dramatic inhibition of subsequent $^{125}$I-transferrin binding. A 1000-fold excess of bovine serum albumin had no effect on $^{125}$I-transferrin binding.

The results of immunofluorescence studies showed that at physiological concentrations of human transferrin almost all amastigotes bound this protein but trypomastigotes did not (Table I). Controls performed in the absence of transferrin did not show fluorescence associated to parasites. Similar experiments carried out with live preparations of these two mammalian stages of T. cruzi showed the same results (data not shown).

**Binding of $^{59}$Fe-transferrin to and dissociation from amastigotes.** To distinguish cell surface-bound from internalized ligand, we examined the release of $^{59}$Fe-transferrin from amastigotes by acid [11]. These conditions release iron from the iron-transferrin receptor complex in mammalian cells [11]. As shown in Table II, $^{59}$Fe-transferrin, which bound to amastigotes under conditions in which ligand-receptor complex internalization does not occur ($4^\circ$C for 1 hr), was dissociated from the parasite surface. However, $^{59}$Fe-transferrin bound to amastigotes at $37^\circ$C for 1 h was not dissociated by acid treatment.
Identification of human transferrin receptors on amastigotes.

We found that a protein of relative molecular weight of 200 kDa interacted with human transferrin when amastigote membrane preparations were resolved by SDS-PAGE under non-reducing conditions, blotted onto nitrocellulose membranes, probed with the B3/25 monoclonal antibody specific for the human transferrin receptor and incubated with goat anti-mouse IgG labeled with alkaline phosphatase (Fig. 2). No band was present when the blots were incubated with an irrelevant mouse antibody or when blots of trypomastigote membranes obtained as described above were treated with this monoclonal antibody and with goat anti-mouse IgG (Fig 2). This protein is present on the cell surface of amastigotes as indicated by western blots of biotinylated amastigote surface proteins (Fig. 2). Similar results were obtained when western blots of amastigote membrane proteins were probed with ¹²⁵I-transferrin and autoradiographed (results not shown).

Effect of deferroxamine and ferrotransferrin on amastigote growth in cell free-medium. The media used to evaluate amastigote growth was a modified ML-15HA medium [7] without hemin. ML-15HA medium does not contain ferrotransferrin or any source of iron [7]. In addition, amastigotes do not use hemin as a source of iron (Villalta, F. & Lima, F. unpublished observations). In this experiments, media was supplemented with heat inactivated human serum as a source of ferrotransferrin to allow amastigote growth.

Our results indicate that deferroxamine, an iron chelator,
dramatically inhibited amastigote growth with respect to control cultures when it was added to ML-15HA, without hemin, supplemented with 10% human serum (Fig 3). Amastigote growth was restored when deferroxamine was removed from the medium and amastigotes were resuspended in ML-15HA, without hemin, supplemented with 10% human serum (results not shown).

Our results also indicate that ML-15HA supplemented with human serum that had been depleted of ferrotransferrin, was not able to support amastigote growth and that addition of ferrotransferrin restored parasite growth (Fig.4). Addition of apotransferrin to the medium that had been depleted of ferrotransferrin does not restore amastigote growth (results not shown).

DISCUSSION

This study shows that T. cruzi amastigotes present receptors for human transferrin. In the presence of physiological concentrations of ferrotransferrin, this ligand binds to its receptor, which is a 200 kDa protein on amastigotes. Iron, which is essential for amastigote growth, appears to be delivered from ferrotransferrin to amastigotes by receptor mediated endocytosis.

The binding of $^{125}$I-transferrin to amastigotes is concentration dependent, saturable and specific thus meeting the
criteria for a biological receptor. It is interesting that unlike mammalian cells, which present only one type of receptor for transferrin with a Kd of $10^{-9}$M and an apparent molecular weight of 180 kDa [18,19], amastigotes present two classes of receptors with Kds of $10^{-12}$M and an apparent molecular weight of 200 kDa. The parasite receptors therefore seem to have 1000 times higher affinity for transferrin when compared to the host cell counterpart. The fact that trypomastigotes, the invasive but non dividing form of T., does not present receptors for this protein would suggest that receptors for transferrin could be involved in some aspect of parasite differentiation. Transferrin receptors have been associated with differentiation. Normal mammalian cells, for example, express fewer receptors than cancerous and stimulated cells (rapidly proliferating cells) [20]. This has been attributed to the different iron needs of these cells, since iron is needed for cell division [21], RNA polymerases [22] and initiation and maintenance of DNA synthesis [23]. If the absence and presence of transferrin receptors in trypomastigotes and amastigotes, respectively, is also due to the different iron requirements of these forms of the parasite or if the expression of transferrin receptors induces a signal for parasite differentiation is not known.

Amastigotes require iron for their growth in cell-free medium as shown in this work by the ability of deferroxamine, an iron chelator, to suppress parasite growth. Two of our findings
indicate that transferrin could be supplying amastigotes with iron: a) amastigotes are unable to grow in medium supplemented with transferrin-depleted human serum, but grow to control levels when this deficient medium is supplemented with physiological concentrations of ferrotransferrin; b) amastigotes are able to internalize ferrotransferrin as shown by the inability of a mild acid treatment to remove ferrotransferrin bound to amastigotes at 37°C as opposed to its dissociation parallel experiments were performed at 4°C.

Host hypoferric responses occur during infection of mice with T. cruzi [24] and it was speculated that this may be due to the transfer of iron to sites of intracellular parasite multiplication, since depletion of host intracellular iron stores reduces parasite pathogenicity [24]. These in vivo results would agree with our in vitro observations, reported in this paper, and explain the mechanisms by which the multiplicative forms of the parasite take iron from the host. T. cruzi might compete for iron from ferrotransferrin with the host, since patients with Chagas' disease present severe anemia with low levels of iron in plasma [25]. It is possible that the intracellular forms of T. cruzi can take ferrotransferrin from the cytoplasm of the cell in the early steps of ferrotransferrin-host cell receptor internalization. Amastigotes could capture ferrotransferrin from vesicles containing ferrotransferrin-receptor complex, since this form of the parasite which is free in the cytoplasm can secrete
proteases [26] and may digest these vesicles. Alternatively, amastigotes could capture ferrotransferrin would be when these forms of the parasite are released from bursting infected cells. These two possibilities are currently under investigation in our laboratory.

Since *T. cruzi* requires an intracellular location to multiply and disseminate in the body, a detailed investigation of peculiar mechanisms by which this intracellular human parasite takes iron from the host could offer a novel approach to blocking the infection.

ACKNOWLEDGMENTS

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REFERENCES


**TABLE I**

**Presence of receptors for human transferrin on the surface of mammalian stages of *T. cruzi***

<table>
<thead>
<tr>
<th>Stage of <em>T. cruzi</em></th>
<th>Parasite pretreatment</th>
<th>Transferrin concentration (ug ml(^{-1}))</th>
<th>Percentage of fluorescent cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amastigote</td>
<td>No</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Amastigote</td>
<td>Yes</td>
<td>1000</td>
<td>75.9 ± 0.4</td>
</tr>
<tr>
<td>Amastigote</td>
<td>Yes</td>
<td>2000</td>
<td>94.8 ± 2.5</td>
</tr>
<tr>
<td>Trypomastigote</td>
<td>No</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Trypomastigote</td>
<td>Yes</td>
<td>1000</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Trypomastigote</td>
<td>Yes</td>
<td>2000</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

This set of results is typically representative of three independent experiments with the same protocol.
<table>
<thead>
<tr>
<th>Temperature of 59Fe-transferrin binding to amastigotes</th>
<th>Mild acid pretreatment of amastigotes containing bound 59Fe-transferrin</th>
<th>Radioactivity associated to amastigotes (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>Yes</td>
<td>282 ± 27^a</td>
</tr>
<tr>
<td>4°C</td>
<td>No</td>
<td>3,993 ± 131^b</td>
</tr>
<tr>
<td>37°C</td>
<td>Yes</td>
<td>4,281 ± 168^c</td>
</tr>
<tr>
<td>37°C</td>
<td>No</td>
<td>5,480 ± 1571^d</td>
</tr>
</tbody>
</table>

59Fe-transferrin was incubated with amastigotes, as described in materials and methods, either at 4°C or 37°C. After removing unbound radioactivity amastigotes were treated with acid for 5 seconds at 4°C, the pH was immediately neutralized to 7.0 and the radioactivity associated to amastigotes was determined. Differences between a and b were statistically significant, but not between c and d (P< 0.05).
FIGURE LEGENDS

Fig. 1. Specific binding of $^{125}$I-labeled human transferrin to T. cruzi amastigotes. Insert is a Scatchard analysis of the binding data. This is a representative experiment from three independent experiments with similar results. Binding assays and analysis of the data were performed as described in Materials and Methods.

Fig. 2. Identification of human transferrin receptors on T. cruzi amastigotes. A, SDS-PAGE of biotinylated surface proteins of T. cruzi amastigotes, blotted onto nitrocellulose membrane, reacted with A-AP and developed. B, SDS-PAGE of T. cruzi amastigote membranes, blotted onto nitrocellulose, probed with monoclonal antibody B$_3$/25 specific for human transferrin receptor and developed. C, SDS-PAGE of T. cruzi amastigote membranes, blotted onto nitrocellulose, probed with an irrelevant monoclonal antibody and developed as above. D, SDS-PAGE of T. cruzi trypomastigote membranes, blotted onto nitrocellulose, probed with monoclonal antibody B$_3$/25 specific for human transferrin receptor and developed as above. Arrows indicate the 200 kDa recognized by the monoclonal antibody B$_3$/25. Biotinylated molecular standards (Bio-Rad) are indicated in kilodaltons.

Fig. 3. Effect of deferroxamine on T. cruzi amastigote growth. Amastigotes were incubated in ML-15HA supplemented with 10% FBS.
or in ML-15HA supplemented with 10% FBS and deferroxamine (○). Amastigote growth was microscopically evaluated as described in Material and Methods.

Fig. 4. *T. cruzi* amastigote growth in medium supplemented with human serum depleted of transferrin and supplemented with ferrotransferrin. A, amastigotes incubated for 48h at 37°C in modified ML-15HA supplemented with transferrin depleted human serum (■). B, amastigotes incubated in modified ML-15HA containing transferrin-depleted human serum supplemented with ferrotransferrin (■). C, amastigotes incubated in modified ML-15HA supplemented with normal human serum (■). The initial amastigote concentration (■) and the parasite concentration after 48h of growth were microscopically determined as described in Material and Methods.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fibronectin Increases *Trypanosoma cruzi* Amastigote Binding to and Uptake by Murine Macrophages and Human Monocytes

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**Materials and Methods**

**Animals.** Crl:CD-1(ICR)BR Swiss mice aged 7 weeks from Charles River Breeding Laboratories, Inc. (Raleigh, N.C.) were used as a source of mouse peritoneal macrophages.

**Parasites.** The Tulahuen strain of *T. cruzi* was used in this work. Blood trypomastigotes isolated from infected mice by chromatography on a diethylaminoethyl-cellulose column (9) were used to infect Vero cell cultures (19). Amastigotes released from these infected cells were isolated on a metrizamide gradient (19) and grown in ML-15HA medium as described previously (19). Amastigotes were also isolated from the spleens of infected mice (9). No enzymes were used in this procedure. The latter amastigotes were purified in a linear sucrose gradient (9) followed by a metrizamide gradient (19). Amastigotes grown in ML-15HA or isolated from the spleens of infected mice were washed by centrifugation with Dulbecco modified minimal essential medium supplemented with 100 U of penicillin and 100 μg of streptomycin per ml ([DMEM] GIBCO Laboratories, Grand Island, N.Y.). To study the interaction between amastigotes and macrophages or monocytes, suspensions of parasites were adjusted to 1 × 10⁷ organisms per ml in DMEM supplemented with 1% bovine serum albumin ([DMEM]-BSA) Sigma Chemical Co., St. Louis, Mo.).

**Radioiodination of fibronectin.** Purified human plasma Fn was obtained from Calbiochem-Behring, San Diego, Calif. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8) of this Fn revealed a sharp band of 220 kilodaltons. Fn (1 mg) was mixed with 2 μCi of [¹²⁵I]Na (specific activity, 17 Ci per milligram of I; ICN Biochemicals, Irvine, Calif.) in Iodogen (Pierce Chemical Co., Rockford, Ill.)-coated tubes (4) at room temperature for 15 min. Unbound radioactivity was removed by gel filtration through Sephadex G-25 (Pharmacia, Inc., Piscataway, N.J.). Radiolabeled Fn was concentrated by ultrafiltration in Centrisart I tubes (Vanguard International, Inc., Neptune, N.J.). The specific activity of the concentrated [¹²⁵I]-labeled Fn was 1 × 10⁶ to 2 × 10⁶ cpm/μg.

**Binding assays.** Triplicate groups of Eppendorf tubes (1.5 ml) precoated with 20% BSA received 50-μl portions of the amastigote suspension (4 × 10⁶ amastigotes/ml in phosphate-buffered saline [pH 7.2] plus 1% BSA) followed by 50 μl of phosphate-buffered saline–BSA containing increasing concentrations of [¹²⁵I]-labeled Fn (0.9 to 30.0 μg/ml in phosphate-buffered saline–BSA) and 50 μl of phosphate-buffered saline–BSA alone or containing 100-fold excess unlabeled Fn to determine nonspecific binding. The tubes were incubated at room temperature with constant shaking for 1 h. Unbound radiolabeled Fn was removed by centrifugation at 4°C.
Specific binding was determined by subtracting nonspecific binding from the total amount of counts bound.

Mouse peritoneal macrophage and human blood monocyte cultures. Mice were killed by excess ether anesthesia and then injected intraperitoneally with 3 ml of sterile DMEM supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) (DMEM + FBS) containing 10 U of heparin per ml. The methods for collecting and processing the peritoneal cells and for preparing resident mouse peritoneal macrophage monolayers have been described (24). Human mononuclear cells were obtained from blood by density gradient centrifugation on Sepacell-MN (Sepacell Corporation, Oklahoma City, Okla.) using the whole blood separation procedure described by the manufacturer. The band containing mononuclear cells was washed in DMEM and suspended in DMEM-fetal bovine serum, and human blood monocytes were separated by adherence to the bottom of Lab-Tek tissue culture chambers (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) for 2 h. Macrophages and monocyte monolayers consisted of > 98% nonspecific esterase-positive cells with typical macrophage morphology. Adherent mouse peritoneal macrophages were further incubated overnight in DMEM-fetal bovine serum at 37°C in an atmosphere of 5% CO₂, whereas human blood monocytes were incubated for 2 h under similar conditions before the parasites were added.

Measurement of amastigote binding to macrophages. Mouse peritoneal macrophage monolayers were washed with DMEM at 4°C immediately prior to adding Fn and untreated or Fn-treated parasites suspended in DMEM-BSA. The parasite:macrophage ratio used was 6:1. The cocultures were incubated at 4°C for 3 h, and the nonbound amastigotes were removed by washing with cold DMEM. After fixation with absolute methanol and staining with Giemsa, the percentage of cells containing bound T. cruzi amastigotes and the number of amastigotes bound per 100 cells were microscopically determined. Independent experiments were performed in triplicate.

Measurement of amastigote uptake by mouse peritoneal macrophages or human blood monocytes. The procedure to measure amastigote uptake by human blood monocytes and mouse peritoneal macrophages has been described (21, 24). Briefly, mouse peritoneal macrophages or human blood monocyte monolayers were washed with DMEM. These cultures then received 100 µl of amastigote suspension in DMEM-BSA and 100 µl DMEM-BSA or DMEM-BSA supplemented with selected concentrations of Fn, goat F(ab')₂ fragment of anti-human Fn immunoglobulin G (IgG) (Cooper Biomedical, Inc, Malvern, Penn.), goat F(ab')₂ fragment of anti-human IgG (Sigma, St. Louis, Mo.), or a combination of these agents. The cocultures were incubated in 5% CO₂ at 37°C for 2 h. Under these conditions, the majority of cell-bound amastigotes were ingested by macrophages (21, 24, 25) or monocytes (21), as further confirmed by electron microscopy studies (results not shown). The unbound parasites were removed by washing each culture chamber with DMEM. The cocultures were fixed, stained, and counted as described previously.

Pretreatment of amastigotes and macrophages with Fn or RGDS. Amastigotes or macrophages were washed with DMEM and preincubated at 4°C for 1 h with DMEM-BSA or DMEM-BSA containing Fn (200 µg/ml). In some experiments, amastigotes were also pretreated with the RGDS tetrapeptide (2 mg/ml in DMEM-BSA) of the Fn cell attachment site (CalBiochem). Amastigotes and macrophages were then washed with DMEM. The parasite suspension was adjusted to a concentration of 1 x 10⁷ organisms per ml in DMEM-BSA and incubated with Fn-treated or untreated macrophage counterparts. Amastigotes that were pretreated with the RGDS tetrapeptide were added to macrophage or monocyte cultures containing DMEM-BSA or DMEM-BSA supplemented with Fn (200 µg/ml). Experiments were conducted as described previously. The selected concentration of RGDS did not affect the ability of macrophages to exclude trypan blue and ingest latex beads. In addition, this concentration of RGDS did not affect the morphology of the parasite... values and represent two to five independent experiments with similar results. Binding assays and analysis of the data were performed as described in Materials and Methods.

RESULTS

Binding of radiolabeled Fn to T. cruzi amastigotes. The binding of radiolabeled human Fns to T. cruzi amastigotes was specific and saturable, indicating the presence of Fn receptors on this form of the parasite (Fig. 1). As indicated in the insert of Fig. 1, Scatchard analysis (17) of the binding data indicates that there is a single class of receptors for Fn present at 1.33 x 10⁵ per amastigote with a Kₘ of 2.3 nM. The RGDS tetrapeptide at the concentration of 4.6 mM inhibits 50% of the binding of ¹²⁵I-labeled Fn to amastigotes.

Effect of Fn on the binding to and uptake of T. cruzi amastigotes by murine macrophages and human blood monocytes. In binding experiments performed at 4°C, pretreatment of either macrophages or amastigotes with Fn (Table 1)
or addition of Fn to amastigote-macrophage cocultures (Table 2) resulted in increased binding of amastigotes to macrophages, as evidenced by a significant increase in both the percentage of macrophages containing bound amastigotes and the number of amastigotes bound to 100 macrophages. Furthermore, the increase in cell binding observed in the presence of Fn was specifically blocked by an anti-human Fn F(ab')2 fragment but not by an anti-human F(ab')2 IgG fragment (Table 2).

Experiments designed to evaluate the uptake of amastigotes by macrophages and monocytes were performed at 37°C. The addition of different concentrations of Fn to macrophage-amastigote cocultures resulted in increased uptake of parasites by macrophages, as evidenced by a significant increase in both the percentage of macrophages containing amastigotes and the number of amastigotes bound to 100 macrophages. Furthermore, the increase in cell binding observed in the presence of Fn was specifically blocked by an anti-human Fn F(ab')2 fragment but not by an anti-human F(ab')2 IgG fragment (Table 2).

The ability of macrophages to take up amastigotes varied occasionally between different experiments of similar proto-

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The ability of macrophages to take up amastigotes varied occasionally between different experiments of similar proto-

Pretreatment of either macrophages or amastigotes with Fn also caused a significant increase in the uptake of parasites by macrophages (Table 4). Furthermore, the enhancing effect of Fn on amastigote uptake by macrophages could be inhibited by an anti-human Fn F(ab')2 fragment but not by an anti-human IgG F(ab')2 fragment (Table 5). The presence of either F(ab')2 fragment in the absence of Fn did not significantly alter parasite-host cell association with respect to control values (Table 5). Pretreatment of amastigotes with the tetrapeptide RGDS (that contains the Fn cell attachment site) (15, 16, 27) inhibited the enhancing effect of Fn on parasite uptake by macrophages (Table 6).

In the presence of Fn, human blood monocytes showed a significant increase in their ability to take up amastigotes (Table 7). The ability of Fn to enhance amastigote uptake by monocytes was inhibited when amastigotes were pretreated with RGDS. In the absence of Fn, RGDS had no effect on the ability of monocytes to take up amastigotes with respect to control values (Table 7).

The ability of macrophages to take up amastigotes isolated from the spleens of infected mice was significantly enhanced by Fn. In a representative experiment, addition of Fn caused a statistically significant increase in both the percentage of macrophages containing amastigotes (24.8 ± 0.5) and the number of amastigotes per 100 macrophages (27.6 ± 0.5) when these values were compared with control values (16.8 ± 1.6 and 18.4 ± 1.1, respectively). The enhancing effect of Fn in these experiments was similar to that observed in

### Table 1: Pretreatment of Either T. cruzi Amastigotes or Macrophages with Fn Increases Binding of Amastigotes to Macrophages

<table>
<thead>
<tr>
<th>Cell and pretreatment</th>
<th>% Macrophages associated with amastigotes</th>
<th>No. of amastigotes bound per 100 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-BSA</td>
<td>24.6 ± 2.9</td>
<td>23.8 ± 1.6</td>
</tr>
<tr>
<td>Fn</td>
<td>33.3 ± 1.8 (35.4)</td>
<td>36.3 ± 6.0 (40.7)</td>
</tr>
<tr>
<td>DMEM-BSA</td>
<td>25.6 ± 1.6</td>
<td>27.2 ± 1.3</td>
</tr>
<tr>
<td>Fn</td>
<td>34.6 ± 1.9 (35.2)</td>
<td>39.8 ± 2.6 (46.3)</td>
</tr>
</tbody>
</table>

* Amastigotes and macrophages were each pretreated with either DMEM-BSA or Fn (200 μg/ml in DMEM-BSA), washed with DMEM, and incubated with their counterparts at 4°C for 3 h.

### Table 2: Anti-Fn F(ab')2 Specifically Inhibits the Enhancing Effect of Fn on the Binding of Amastigotes to Macrophages

<table>
<thead>
<tr>
<th>Coculture conditions</th>
<th>% Macrophages containing amastigotes</th>
<th>No. of amastigotes bound per 100 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-BSA</td>
<td>17.1 ± 0.1</td>
<td>19.0 ± 1.6</td>
</tr>
<tr>
<td>Fn</td>
<td>23.7 ± 0.1* (35.7)</td>
<td>24.3 ± 0.7* (27.9)</td>
</tr>
<tr>
<td>Anti-Fn F(ab')2</td>
<td>17.1 ± 0.2</td>
<td>18.5 ± 0.4 (2.6)</td>
</tr>
<tr>
<td>Fn + anti-Fn F(ab')2</td>
<td>18.2 ± 1.3</td>
<td>19.7 ± 0.1 (3.7)</td>
</tr>
<tr>
<td>Anti-IgG F(ab')2</td>
<td>17.4 ± 1.1*</td>
<td>19.1 ± 2.0 (2.5)</td>
</tr>
<tr>
<td>Fn + anti-IgG F(ab')2</td>
<td>21.5 ± 1.3* (37.5)</td>
<td>23.4 ± 0.6* (23.2)</td>
</tr>
</tbody>
</table>

* Fibronectin, antibodies, or both were added to amastigote-macrophage cocultures and incubated at 4°C for 3 h.

### Table 3: Addition of Exogenous Fn to Macrophage-T. cruzi Amastigote Cocultures Increases Parasite Uptake by Macrophages

<table>
<thead>
<tr>
<th>Fn (μg/ml)</th>
<th>% Macrophages containing amastigotes</th>
<th>No. of amastigotes per 100 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>16.8 ± 3.1</td>
<td>28.3 ± 3.5</td>
</tr>
<tr>
<td>25.0</td>
<td>25.9 ± 2.3 (42.4)*</td>
<td>37.1 ± 3.9 (31.1)*</td>
</tr>
<tr>
<td>100.0</td>
<td>27.0 ± 2.3 (60.7)*</td>
<td>42.3 ± 2.9 (49.5)*</td>
</tr>
<tr>
<td>200.0</td>
<td>32.7 ± 3.2 (94.6)*</td>
<td>49.6 ± 1.1 (75.3)*</td>
</tr>
</tbody>
</table>

* Amastigotes were added to macrophage monolayers containing different concentrations of Fn and incubated at 37°C for 2 h.

### Table 4: Pretreatment of Either T. cruzi Amastigotes or Macrophages with Fn Increases the Uptake of Amastigotes by Macrophages

<table>
<thead>
<tr>
<th>Cell and pretreatment</th>
<th>% Macrophages containing amastigotes</th>
<th>No. of amastigotes per 100 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-BSA</td>
<td>20.1 ± 2.2</td>
<td>25.3 ± 2.2</td>
</tr>
<tr>
<td>Fn</td>
<td>26.9 ± 1.8 (33.8)*</td>
<td>33.0 ± 0.6 (30.4)*</td>
</tr>
<tr>
<td>DMEM-BSA</td>
<td>20.2 ± 1.3</td>
<td>24.4 ± 2.4</td>
</tr>
<tr>
<td>Fn</td>
<td>25.2 ± 0.4 (24.8)*</td>
<td>32.8 ± 2.1 (34.4)*</td>
</tr>
</tbody>
</table>

* Amastigotes and macrophages were each pretreated with either DMEM-BSA or Fn (200 μg/ml in DMEM-BSA), washed with DMEM, and exposed to their counterparts at 37°C for 2 h.

* Mean ± standard deviation of triplicate cocultures of a representative experiment.

* Differences between these values and the corresponding control values were statistically significant (P ≤ 0.05). Percent change with respect to control values is given in parentheses.
TABLE 5. Anti-human Fn F(ab')2 fragment specifically inhibits the enhancing effect of Fn on the uptake of T. cruzi amastigotes by macrophages

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% Macrophages containing amastigotes</th>
<th>No. of amastigotes per 100 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-BSA</td>
<td>19.7 ± 1.7</td>
<td>22.0 ± 3.4</td>
</tr>
<tr>
<td>Fn</td>
<td>30.6 ± 0.1 (55.3)</td>
<td>40.0 ± 0.1 (81.8)</td>
</tr>
<tr>
<td>Anti-Fn F(ab')2</td>
<td>18.8 ± 2.9 (-4.0)</td>
<td>22.0 ± 2.5 (0.0)</td>
</tr>
<tr>
<td>Fn + anti-Fn F(ab')2</td>
<td>18.3 ± 0.6 (-7.1)</td>
<td>22.1 ± 0.5 (0.5)</td>
</tr>
<tr>
<td>Anti-lgG F(ab')2</td>
<td>20.4 ± 1.6 (3.6)</td>
<td>24.8 ± 5.5 (12.7)</td>
</tr>
<tr>
<td>Fn + anti-lgG F(ab')2</td>
<td>27.2 ± 4.0 (38.1)</td>
<td>33.4 ± 1.8 (51.8)</td>
</tr>
</tbody>
</table>

* Fn, antibodies, or both were added to amastigote-macrophage cocultures, and the cocultures were incubated at 37°C for 2 h.
* Mean ± standard deviation of triplicate cocultures of a representative experiment. Percent change with respect to control values is given in parentheses.
* Differences between these values and the corresponding control values (DMEM-BSA) were statistically significant (P < 0.05).

DISCUSSION

This study shows that T. cruzi amastigotes present receptors for human fibronectin (Fig. 1). In the presence of physiological concentrations of fibronectin, these receptors appear to increase the binding to and uptake of amastigotes by both murine macrophages and human monocytes. The enhancing effect of Fn on amastigote binding to and uptake by these two types of phagocytic cells appears to be specific since anti-human Fn F(ab')2 fragments can inhibit it (Tables 1 and 5), apparently by interacting with the Fn molecule.

The fact that pretreatment of either macrophage or T. cruzi amastigotes with Fn intensifies the degree of parasite binding to and uptake by macrophages indicates that Fn receptors on both amastigotes (as stated previously) and macrophages (27) are responsible for the effect of Fn on amastigote-phagocytic cell interactions. This finding suggests that T. cruzi amastigote binding to and uptake by mononuclear phagocytic cells is mediated in part by the interaction of amastigote-bound Fn with macrophage and monocyte Fn receptors or vice versa by a bridging phenomenon. However, since anti-human Fn F(ab')2 fragment does not significantly affect the binding and uptake of amastigotes in the absence of Fn, it is likely that Fn is not the only ligand involved in T. cruzi amastigote-macrophage or amastigote-monocyte interactions.

The fact that pretreatment of T. cruzi amastigotes with RGDS inhibits the effect of Fn on amastigote uptake by either murine macrophages or human monocytes (Tables 6 and 7) suggests that the enhancing effect of Fn is mediated by the Fn cell attachment site (RGDS). Macrophage uptake of amastigotes isolated from the spleens of infected mice is also enhanced by Fn, suggesting that this interaction may occur in vivo.

TABLE 6. Pretreatment of T. cruzi amastigotes with RGDS inhibits the enhancing effect of Fn on amastigote uptake by macrophages

<table>
<thead>
<tr>
<th>Amastigote pretreatment (addition of Fn to cocultures)</th>
<th>% Macrophages containing amastigotes</th>
<th>No. of amastigotes per 100 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-BSA (-)</td>
<td>9.8 ± 0.1</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td>DMEM-BSA (+)</td>
<td>19.8 ± 2.9 (102.0)</td>
<td>20.8 ± 3.5 (108.0)</td>
</tr>
<tr>
<td>RGDS (-)</td>
<td>7.5 ± 1.1 (-23.5)</td>
<td>7.3 ± 1.3 (-27.0)</td>
</tr>
<tr>
<td>RGDS (+)</td>
<td>7.7 ± 1.1 (-21.4)</td>
<td>7.9 ± 0.8 (-21.0)</td>
</tr>
</tbody>
</table>

* Amastigotes were preincubated either with DMEM-BSA or RGDS (2 mg/ml in DMEM-BSA) for 1 h at 4°C and, after washing, were added to macrophage cultures containing DMEM-BSA or Fn (200 µg/ml in DMEM-BSA).
* Symbols indicate addition (+) or no addition (-) of Fn.
* Mean ± standard deviation of triplicate cocultures of a representative experiment.
* Differences between these values and the corresponding control values (DMEM-BSA) were statistically significant (P < 0.05). Percent change with respect to control values is given in parentheses.

The observation that inflammatory cells surrounding tissue lesions contain amastigotes (7) and the fact that macrophages, monocytes, eosinophils, and neutrophils take up and destroy these forms of the parasite released from mammalian cells (20–23) indicate that these cells may be involved in the reduction of tissue forms of the parasite observed in the chronic phase of the disease. Recent observations indicate that when pure populations of amastigotes growing in logarithmic phase are ingested by inflammatory cells, these forms of the parasite are destroyed and digested (20–23). When macrophages ingest amastigotes contaminated with small amounts of transitional forms in differentiation from amastigotes to trypomastigotes or trypomastigotes, macrophages destroy and digest amastigotes, but the few transi-
ROLE OF T. CRUZI AMASTIGOTE RECEPTORS FOR FIBRONECTIN

Our findings that T. cruzi amastigotes present receptors for Fn, together with the observations of others that trypanomastigote (11, 26) and epimastigote (13) forms of the same parasite present receptors for this molecule, indicate that Fn receptors are present on all stages of this parasite, regardless of their invasive capacities. This molecule may therefore play a significant role in increasing the attachment and incorporation of intracellular forms of T. cruzi, released from bursting infected cells, by macrophages and monocytes at the level of chagasic tissue lesions.

ACKNOWLEDGMENTS

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