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Characterization of the Fibronectin Binding Properties of *Trypanosoma cruzi* Epimastigotes

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ABSTRACT

Interactions between parasites and host cells play an important role in the infection process. The adhesive protein fibronectin has been shown to be involved in the interaction between *Trypanosoma cruzi* and host cells. *Trypanosoma cruzi* epimastigotes grown in axenic culture bound ^{125}I-fibronectin in an energy-dependent manner. Initial binding induced the expression/activation of further fibronectin receptors or activation of a fibronectin-specific uptake system. This binding could be inhibited by fixing the parasites with formalin, metabolic inhibitors (e.g. dinitrophenol), changes in temperature and by an excess of unlabeled fibronectin. The binding was not inhibited by EDTA nor by the peptide Arg-Gly-Asp-Ser (RGDS), which is the recognition sequence from the major cell binding domain of fibronectin.

INTRODUCTION

Trypanosoma cruzi is an insect-borne parasite found mainly in Central and South America. It is usually transmitted by the faeces of an infected triatomine insect (Reduviidae). The main pathological consequence is Chagas' disease which affects the heart. There are an estimated 16-18 million people infected and existing treatment is not very effective (Brener, 1973; WHO Expert Committee, 1991).

In the insect vector, *T. cruzi* has 2 stages of development. One is the infectious, non-dividing metacyclic stage and the other is a non-infectious, dividing epimastigote stage. In humans it also has 2 stages both of which are infectious. One is the non-dividing, extracellular trypomastigote stage and the other is the replicating, intracellular amastigote stage (Brener, 1973). Since only the intracellular stage can replicate, cell invasion is an important step in the life

cycle. *Trypanosoma cruzi* trypomastigotes have been shown to invade fibroblasts (Ouaissi et al., 1985) and heart myoblasts (Villalta et al., 1990), and amastigotes have been shown to invade macrophages and monocytes (Noisin and Villalta, 1989), while epimastigotes can invade macrophages only (Rimoldi et al., 1989). Attachment to the target cell is the first stage in the invasion process. Inhibition of attachment interrupts the life cycle and by maintaining the parasite in the blood allows the immune system greater access to the parasites.

The adhesive protein fibronectin plays an important role in the attachment of parasites to host cells and has been shown to play a role in *T. cruzi* trypomastigote attachment to host cells (Ouaissi et al., 1985; Wirth and Kierszenbaum, 1984). Studies on the cell attachment domains of fibronectin showed that in the major cell-binding domain, the minimum sequence for recognition was the tetrapeptide Arg-Gly-Asp-Ser (RGDS) (Pierschbacher and Ruoslahti, 1984b). This peptide and its analogues have been shown to inhibit platelet aggregation (Ruggeri et al., 1986) and cell adhesion (Pierschbacher and Ruoslahti, 1984a). It has also been shown to play a role in the attachment of fibronectin to *T. cruzi* trypomastigotes (Ouaissi et al., 1986), amastigotes (Noisin and Villalta, 1989) and epimastigotes (Kanbara et al., 1986).

Since epimastigotes can easily be grown in axenic culture, we decided to study the fibronectin binding properties of *T. cruzi* epimastigotes to determine if they can be used instead of trypomastigotes in binding studies for inhibitors of fibronectin binding. In particular, we studied the actions of RGDS which has been shown to inhibit fibronectin binding to trypomastigotes. Attachment of *T. cruzi* to cells has been shown to be dependent on parasite energy (Schenkman et al., 1991), so we investigated the role of parasite energy production on fibronectin binding. We also looked at the inhibitory effects of pentamidine which is an anti-parasite agent that was originally developed for treatment of infections by *T. rhodesiense*, *T. gambiense* and *Leishmania donovani* and currently, in the treatment of *Pneumocystis carinii* pneumonia (Goa and Campoli-Richards, 1987). We have previously shown that pentamidine is an inhibitor of platelet GPIIb/IIIa (Cox et al., 1992) which is an RGD-dependent receptor for fibrinogen.

MATERIALS AND METHODS

Cultures and reagents: *Trypanosoma cruzi* (Tulahuen strain) was a gift from Dr. H. Kanbara, Dept. of Protozoology, Institute of Tropical Medicine, Nagasaki University, Japan. Pentamidine and RGDS were synthesized by the Fujisawa Chemistry Department (>98% pure). Tryptose was purchased from Difco (USA), liver broth from Oxoid (UK) and DE52 cellulose from Whatman (UK). Hemin, HEPES and fibronectin were obtained from Sigma, USA. Penicillin /streptomycin and Minimum Essential Medium (MEM) were obtained from Flow Laboratories, Scotland and foetal calf serum (FCS) was from Cell Culture

Technologies, USA. $Na^{125}I$ was from Amersham (UK) and EDTA was from Hayashi, Japan. All other reagents were purchased from Ishizu Pharmaceutical Company, Japan.

Isolation of *T. cruzi*: Six mice (ICR, albino) were inoculated with *T. cruzi* trypomastigotes from frozen stocks. Parasitemia was monitored by serial tail bleeding and counting by microscope. All subsequent procedures were performed aseptically. When parasitemia reached a peak the mice, under ether anesthesia, were bled into heparin. The blood was incubated with 6% (w/v) dextran for 45 min at room temperature followed by centrifugation at 60xg for 20 min (De Titto et al., 1986). The parasite-rich supernatant was centrifuged to pellet the cells and resuspended in culture medium. The parasite suspension was then passed over a DE52 cellulose column to remove contaminating cells. The yield of cells was $6.0x10^6$ parasites and $3.3x10^8$ blood cells before chromatography and $2.6x10^6$ parasites and $6.0x10^5$ blood cells after chromatography. The parasites were then adjusted to $1.7x10^6$/ml in culture medium.

Culture of *T. cruzi*: The medium used for culture was Liver infusion Tryptose (Bone and Steinert,1956) which contains 4g NaCl, 5g $Na_3PO_4.12H_2O$, 0.4g KCl, 2g glucose, 15g tryptose and 3g liver broth, pH 7.8, in 1litre. This was stirred for 30 min, filtered and autoclaved. Before use, it was supplemented with 20 µg/ml hemin (100x stock in 0.1 M NaOH, and autoclaved), 20% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin (LIT medium). The trypomastigotes isolated from blood were incubated at room temperature (RT, 25°C) in LIT. After a few days, the trypomastigotes transformed into the amastigote stage. The amastigotes were easily cultured in large numbers in LIT by changing the medium every 2 days. Amastigote cultures that were left without feeding, i.e., aged for about 1 week, transformed into epimastigotes (Rondinelli et al., 1988). A pure epimastigote culture could be maintained with regular feeding.

^{125}I-fibronectin binding studies: Fibronectin was labeled by the chloramine-T method (Greenwood et al., 1963). The specific activity was 6mCi/mg. For binding studies, epimastigotes were suspended in either MEM or HEPES-Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3 mM NaH_2PO_4, 3.5 mM HEPES, 5.5 mM glucose, pH 7.5, containing 0.35% w/v bovine serum albumin) at a concentration of around $1x10^8$ parasites/ml. The ^{125}I-fibronectin concentration was 2 µg/ml. For inhibition studies, live parasites or parasites fixed with 1% (v/v) formalin (30 min at RT) and washed with buffer, were incubated with drug for 30 min at RT, on ice or at 37°C. For time course studies, ^{125}I-fibronectin was added to all tubes at T=0 and at the indicated time intervals, 1/10 volume of formalin (37% v/v) was added. Separation of free from bound (60 min after the addition of ^{125}I-fibronectin) was achieved by centrifugation of 100 µl of sample on 200 µl of 20% (w/v) sucrose in HEPES-

Tyrodes buffer at 10,000 rpm for 5 min in 0.4 ml reaction tubes (Sarstedt, Germany). The pellet was then cut off with a blade and counted in a γ-counter.

RESULTS AND DISCUSSION

Cation-independent fibronectin binding: Initial experiments with epimastigotes comparing binding of ^{125}I-fibronectin in Tyrode buffer and MEM showed that binding was always higher in Tyrode buffer (Table 1). This difference could be abolished by the addition of 10 mM EDTA, indicating that divalent cations were inhibiting the binding. The use of a Tris-saline buffer, with or without EDTA, produced similar binding to that with Tyrode (data not shown). Since Tyrode buffer contained Ca^{2+} and Mg^{2+}, some divalent cation in MEM other than calcium or magnesium probably inhibited the binding.

Table 1: Effects of buffer (Tyrode (Tyr) and Minimum Essential Medium (MEM)), fixation with formalin, EDTA, and excess unlabeled fibronectin (xs fnct) on ^{125}I-fibronectin binding to *T.cruzi* epimastigotes. Binding is represented as molecules bound/parasite± SEM. The SEM is calculated on the mean of 3 samples for each experiment.

Expt no	Buffer	Treatment	Molecules/ Parasite
1	MEM	fixed	387±2
	MEM+EDTA	fixed	710±10
	Tyr	fixed	733±44
	Tyr+EDTA	fixed	662±7
	Tyr+xs fnct	fixed	513±2
2	Tyr	fixed	2,262±33
	Tyr+xs fnct	fixed	1,699±12
	Tyr	unfixed	4,064±6
	Tyr+xs fnct	unfixed	1,514±10

Time-dependent fibronectin binding: Figure 1 shows the time course of fibronectin binding. Epimastigotes were incubated with ^{125}I-fibronectin for 1 hr at RT in each tube. Formalin was added to fix the epimastigotes at timed intervals. The figure shows that total binding is quite different in each preparation but that binding always increases with time. This was probably due to a difference in the growth phase of the cells. Since each sample was incubated with ^{125}I-fibronectin for 60 min, the difference is unlikely to be due to time required for the binding to reach equilibrium. Rather, the difference may reflect the time necessary to activate or expose the fibronectin receptors on the surface of the epimastigote. It is possible that the addition of formalin disrupted the binding of ^{125}I-fibronectin. However, this is unlikely as parasites that were fixed and washed to remove formalin before binding studies

also bind less than unfixed parasites (Table 1).

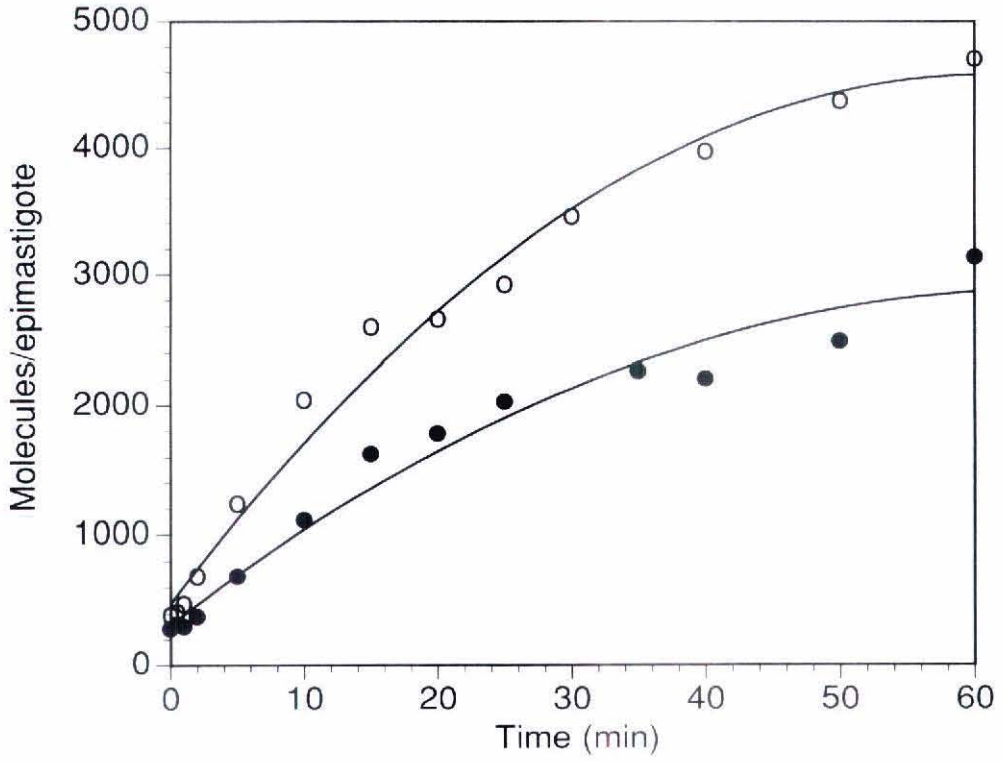

FIG 1: Effect of formalin fixation on the time course of ^{125}I-fibronectin binding to epimastigotes. All tubes were incubated for 60 min with ^{125}I-fibronectin but formalin was added at different stages during the incubation. Each point represents the average of a triplicate from one experiment. Two separate experiments are shown.

Temperature-dependent fibronectin binding: Binding was shown to be temperature dependent (Fig. 2). It was inhibited at both 4°C and 37°C. Inhibition at 4°C probably reflects inhibition of energy dependent processes. Epimastigotes are an insect stage and thus, 37°C is probably not their optimum temperature. Thus, epimastigotes have been shown to decrease total protein synthesis when exposed to temperatures of 37-41°C (Alcina et al., 1988) and to have different surface antigens when culture conditions are increased from 30°C to 34°C (O'Daly and Polanco, 1990).

Energy-dependent fibronectin binding: Studies with formalin-fixed and unfixed epimastigotes showed that while the amount of ^{125}I-fibronectin that bound varied between preparations, unfixed epimastigotes always bound more ^{125}I-fibronectin than fixed epimastigotes (Table 1). This increase was in specific binding only, since the binding in the presence of excess un-

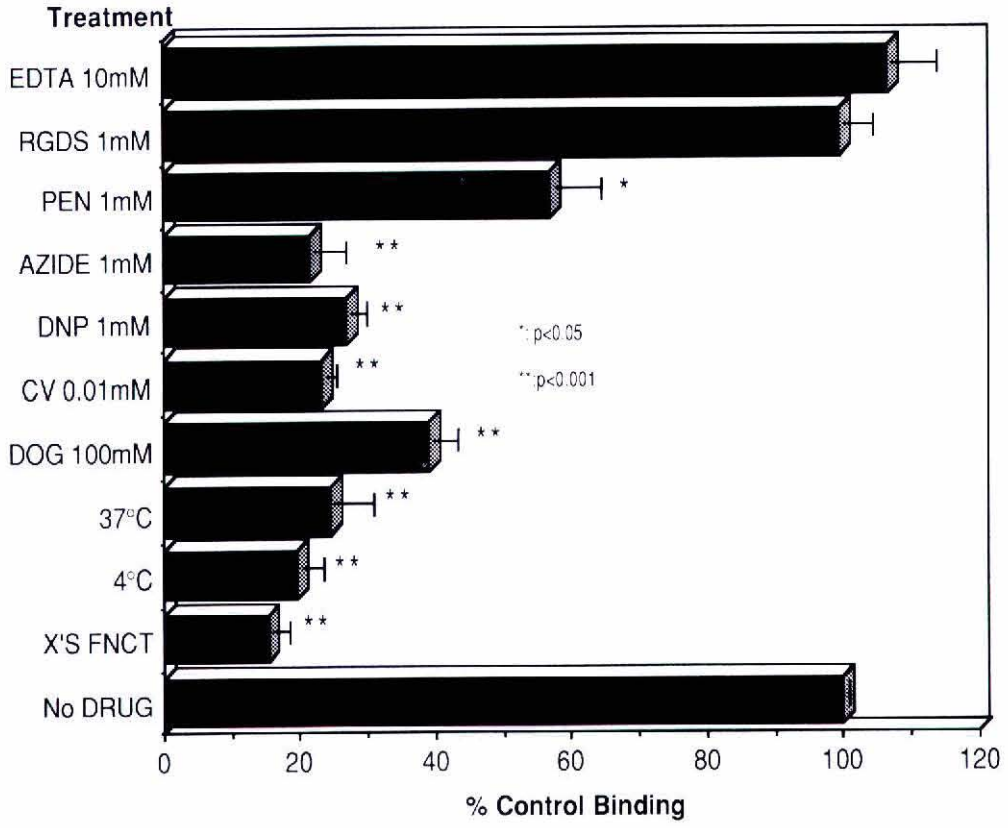

FIG 2: Effect of EDTA, tetrapeptide RGDS, pentamidine (PEN), sodium azide (AZIDE), dinitrophenol (DNP), crystal violet (CV), 2-deoxyglucose (DOG), 100-fold excess of unlabeled fibronectin (X S FNCT) and, incubation at 37°C and 4°C on ^{125}I-fibronectin binding to *T. cruzi* epimastigotes. Error bars indicate the SEM of 3 separate experiments. p value was calculated with respect to the non-drug control using a t-test.

labeled fibronectin was similar in both fixed and unfixed parasites. Binding was inhibited by >80% with a 100-fold excess of unlabelled fibronectin. In unfixed epimastigotes, binding increased with time, indicating an activation or exposure of the fibronectin receptor. These changes in the receptor are dependent on a metabolically active parasite. The process is energy dependent as it is inhibited by formalin. Crystal violet (CV), which acts on the mitochondrion (Gadelha et al., 1989) and dinitrophenol (DNP), which uncouples oxidative phosphorylation in mitochondria also inhibited binding. Sodium azide (AZIDE) and 2-deoxyglucose (DOG), an inhibitor of glycolysis, also inhibited binding (Fig. 2). It is unlikely that synthesis of new receptor is involved since the time scale is so short. Thus, it is due either to activation of the fibronectin receptors, or transport of new receptors to the surface. Another possibility is that there

is an energy-dependent fibronectin uptake system in epimastigotes. The pattern of inhibition of fibronectin binding reported here is similar to the inhibition of *T. cruzi* trypomastigote attachment to mammalian cells (Schenkman et al., 1991). Schenkman and co-workers indicated that some unknown energy dependent process in the parasite is involved. Our data suggest that this energy dependent process may be the activation/expression of the fibronectin receptor or of a specific uptake system.

RGD-independent fibronectin binding: In recent years, there has been a lot of research on cell adhesion molecules. In particular, fibronectin can bind to one family of cell adhesion molecules, the integrins (Ruoslahti, 1991). Fibronectin contains a number of cell attachment sites (Aota et al., 1991). It can attach to integrins in an RGD-dependent manner, eg., $\alpha_5\beta_1$ and in an RGD-independent manner, $\alpha_3\beta_1$ (Ruoslahti, 1991). The attachment of amastigotes to macrophages in the presence of fibronectin was inhibited when pre-incubated with RGDS (2mg/ml) (Noisin and Villalta, 1989). *T. cruzi* trypomastigotes were shown to bind fibronectin in an RGD-dependent manner (Ouaissi et al., 1986). Our data showed that RGDS (1 mM) had no effect on fibronectin binding to epimastigotes, while pentamidine inhibited it, but only at very high concentrations (Fig 2). In a similar assay, RGDS and pentamidine inhibited ^{125}I-fibronectin binding to platelets with IC_{50} values of 42 μ M and 0.16 μ M, respectively (Cox et al., 1992). Also, RGD-dependent receptors are usually cation-dependent (Ruoslahti and Pierschbacher, 1987).

Thus, *T. cruzi* epimastigotes bind fibronectin in an RGD-independent manner. This binding induces the activation/expression of the fibronectin receptor on the surface or activation of a specifc fibronectin uptake system, through an energy-dependent mechanism. This suggests that epimastigotes bind fibronectin in a different manner to trypomastigotes and amastigotes. As epimastigotes are a non-infectious form, the acquisition of RGD-dependent fibronectin binding appears to be an important step in the transformation to the infectious forms.

REFERENCES

Alcina, A., Urzainqui, A. & Carrasco, L. 1988. The heat shock response of *Trypanosoma cruzi*. *Eur. J. Biochem. 172:* 121-127.

Aota, S., Nagai, T. & Yamada, K. 1991. Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mutagenesis. *J. Biol. Chem. 266:* 15938-15943.

Bone, G. J. & Steinert, M. 1956. Isotopes incorporated in the nucleic acids of *Trypanosoma mega*. *Nature: 178,* 308-309.

Brener, Z. (1973). Biology of *Trypanosoma cruzi*. *Ann. Rev. Microbiol. 27:* 347-382.

Cox, D., Motoyama, Y., Seki, J., Aoki, T., Dohi, M. & Yoshida, K. 1992. Pentamidine: A non-peptide GPIIb/IIIa antagonist- in vitro studies on platelets from humans and other species. *Thromb. Haemostas.* In Press.

De Titto, E. H., Catterall, J. R. & Remington, J. S. 1986. Activity of recombinant tumor necrosis factor on *Toxoplasma gondii* and *Trypanosoma cruzi*. *J. Immunol. 137:* 1342-1345.

Gadelha, F. R., Moreno, S. N., DeSouza, W., Cruz, F. S. & Docampo, R. 1989. The mitochondrion of *Trypanosoma cruzi* is a target of crystal violet toxicity. *Mol. Biochem. Parasitol. 34:* 117-126.

Goa, K. L. & Campoli-Richards, D. M. 1987. Pentamidine Isethionate A review of its antiprotozoal activity, pharmacokinetic properties and therapeutic use in *Pneumocystis carinii* pneumonia. *Drugs 33:* 242-258.

Greenwood, F. C., Hunter, W. M. & Glover, J. S. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J. 89:* 114-123.

Kanbara, H., Kondo, G. & Yanagi, T. 1986. Fibronectin attachment to different stages of *Trypanosoma cruzi*. *Trop. Med. 28:* 123-127.

Noisin, E. L. & Villalta, F. 1989. Fibronectin increases *Trypanosoma cruzi* amastigote binding to and uptake by murine macrophages and human monocytes. *Infect. Immun. 57:*1030-1034.

O'Daly, J. A. & Polanco, N. 1990. Variability of *Trypanosoma cruzi* epimastigote surface antigens with changes in the temperature of the cultures. *Am. J. Trop. Med. Hyg. 43:* 44-51.

Ouaissi, M. A., Cornette, J., Afchain, D., Capron, A., Gras-Masse, H. & Tartar, A. 1986. *Trypanosoma cruzi* infection inhibited by peptides modeled from a fibronectin cell attachment domain. *Science 234:* 603-607.

Ouaissi, M. A., Cornette, J. & Capron, A. 1985. *Trypanosoma cruzi*: modulation of parasite-cell interaction by plasma fibronectin. *Eur. J. Immunol. 15:* 1096-1101.

Pierschbacher, M. & Ruoslahti, E. 1984a. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc. Natl. Acad. Sci. (USA) 81:* 5985-5988.

Pierschbacher, M. D. & Ruoslahti, E. 1984b. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature 309:* 30-33.

Rimoldi, M. T., Tenner, A. J., Bobak, D. A. & Joiner, K. A. 1989. Complement component C1q enhances invasion of human mononuclear phagocytes and fibroblasts by *Trypanosoma cruzi* trypomastigotes. *J. Clin. Invest. 84:* 1982-1989.

Rondinelli, E., Silva, R., De Oliveira Carvalho, J. F., De Almeida Soares, C. M., De Carvalho, E. F. & De Castro, F. T. 1988. *Trypanosoma cruzi*: An in vitro cycle of cell differentiation in axenic culture. *Exp. Parasitol. 66:* 197-204.

Ruggeri, Z. M., Houghten, R. A., Russell, S. R. & Zimmerman, T. S. 1986. Inhibition of platelet function with synthetic peptides designed to be high-affinity antagonists of fibrinogen binding to platelets. *Proc. Natl. Acad. Sci.*

(USA) 83: 5708-5712.

Ruoslahti, E. 1991. Integrins. *J. Clin. Invest. 87:* 1-5.

Ruoslahti, E. & Pierschbacher, M. D. 1987. New perspectives in cell adhesion: RGD and Integrins. *Science 238:* 491-497.

Schenkman, S., Robbins, E. S. & Nussenzweig, V. 1991. Attachment of *Trypanosma cruzi* to mammalian cells requires parasite energy and invasion can be independent of the target cell cytoskeleton. *Infect. Immun. 59:* 645-654.

Villalta, F., Lima, M. F. & Zhou, L. 1990. Purification of *Trypanosoma cruzi* surface proteins involved in adhesion to host cells. *Biochem. Biophys. Res. Comm. 172:* 925-931.

WHO Expert Committee 1991. Control of Chagas' disease (WHO Technical Report series No. 811). World Health Organization.

Wirth, J. J. & Kierszenbaum, F. 1984. Fibronectin enhances macrophage association with invasive forms of *Trypanosoma cruzi*. *J. Immunol. 133:* 460-464.