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ROLE OF CELL SURFACE MANNOSE RESIDUES IN HOST CELL INVASION BY *TRYPANOSOMA CRUZI*

FERNANDO VILLALTA and FELIPE KIERSZENBAUM

Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824 (U.S.A.)

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The role of mannose residues on the membranes of *Trypanosoma cruzi* and its host cells in their association (surface binding plus internalization of the parasite) leading to infection was studied. Used in this work were the bloodstream (trypomastigote), intracellular (amastigote) and insect-transmissible (metacyclic trypomastigote) forms of the parasite; mouse macrophages and rat heart myoblasts were used as the host cells. Removal of mannose residues from the surface of all forms of the parasite by treatment with alpha-mannosidase produced a marked increase in their respective abilities to associate with either host cell. The increase was more pronounced with the bloodstream and insect-derived trypomastigotes (which can penetrate cell membranes) than with the amastigotes (which can not do so). By contrast, mannosidase treatment of the macrophages and the myoblasts caused a significant decrease in the ability of these cells to associate with either bloodstream or insect-derived trypomastigote forms. The capacity of mannosidase-treated macrophages to take up the non-invasive amastigotes was also reduced. These results, as a whole, suggest that mannose residues on the surface of the parasite modulate their binding to macrophages and myoblasts and that mannose residues on the surface of these host cells play a role in cell association with the parasite.

Introduction

Trypanosoma cruzi is the unicellular parasite causing Chagas' disease or American trypanosomiasis [1], which afflicts millions of people in South and Central America [2]. The parasite has an intracellular (amastigote, AMA) stage responsible for multiplication in mammalian hosts. Trypomastigote forms occurring in the fecal fluids of the insect vectors and in the blood of infected vertebrates possess the ability to penetrate cell membranes and, thus, initiate the infection in nature and disseminate the disease in the body, respectively (reviewed in Ref. 3). Bloodstream trypomastigotes (BT) derive from the intracellularly dividing AMA forms. Despite the great importance that cell invasion assumes in the establishment of *T. cruzi* infection in mammalian hosts and the relevance of this process to the pathogenesis of Chagas' disease, our understanding of the mechanisms whereby the parasite obtains an intracellular localization remains very limited. Approaches to the clarification of the cell infection process must consider the initial association of the surface of the parasite with that of the host cell. Thus, the identification of parasite membrane components binding host cell surface receptors would go a long way towards improving our

Abbreviations: AMA, amastigote form of *T. cruzi*; BT, blood-derived trypomastigote form of *T. cruzi*; DMEM, Dulbecco's modified MEM; DMEM-BSA, DMEM supplemented with 1% BSA; HSS, Hank's balanced salt solution; mannosidase, alpha-D-mannosidase; MEM, minimal essential medium; MEM-BSA, MEM supplemented with 1% BSA; MFTC, metacyclic forms of *T. cruzi*; PMSF, phenylmethylsulfonyl fluoride.

knowledge of the infection process as well as support the development of effective prevention means. As a part of our efforts in this direction, we are studying the role of surface sugar moieties on both *T. cruzi* and some of its host cells in their ability to associate. Because mannose is present on the surface of all of the life cycle stages of *T. cruzi* [4], has been found in glycoproteins derived from this parasite [5,6] and has been linked to the mechanisms of cell infection by bacteria [7,8], we examined in this work the role of this sugar in the association of invasive and non-invasive forms of *T. cruzi* with different types of host cells.

Materials and Methods

Parasites. The Tulahuén strain of *T. cruzi* was used in this work. The BT forms were isolated from the blood of infected stock Crl : CD-1(ICR)BR Swiss mice (Charles River Laboratories, Portage, MI) by initial separation by density gradient centrifugation over Lymphoprep [9] followed by chromatography on a diethylaminoethyl-cellulose column [10]. The AMA form was grown in ML-15HA medium under conditions described in detail elsewhere [11]. To study interactions between trypomastigote or amastigote forms with macrophages, suspensions of the parasite were adjusted to $1 \cdot 10^7$ organisms per ml in Eagle's minimal essential medium (MEM, Gibco, Grand Island, NY) supplemented with 100 IU penicillin and 100 μg streptomycin per ml and with 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) (MEM-BSA). To study parasite interactions with myoblasts the selected medium for parasite suspension was Dulbecco's modified MEM (DMEM) supplemented with the same amounts of antibiotics and bovine serum albumin as above (DMEM-BSA). Metacyclic forms of *T. cruzi* (MFTC) were isolated from the hindguts of infected insects (*Rhodnius prolixus*) by chromatography over diethylaminoethyl-cellulose [10]. After purification, these flagellates were suspended at the appropriate concentration (see below) in MEM-BSA. Parasite concentrations were determined microscopically using a Neubauer hemacytometer.

Macrophage cultures and their infection. Unelicited, inbred CBA/J mice (Jackson Laboratory, Bar Harbor, ME) were killed by cervical dislocation and then injected 5 ml of sterile MEM supplemented with antibiotics and 5 units/ml heparin intraperitoneally. After gentle abdominal massage, the peritoneal cells were collected and washed twice by centrifugation with MEM. The cells were resuspended at $5 \cdot 10^5$ nucleated cells per ml in MEM supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (FBS, Sterile Systems, Logan, UT). 0.5-ml aliquots of this suspension were placed in wells of Lab-Tek tissue culture slides (Miles Laboratories, Naperville, IL) and incubated at 37°C in a moist 5% CO_2-in-air atmosphere for 2 h. After removal of the nonadherent cells, 0.2 ml of parasite suspension in MEM-BSA containing $1 \cdot 10^6$ enzyme-treated (see below) or untreated parasites was added. The infected cultures were incubated further under the same conditions for variable periods of time up to 2 h. Because small numbers of MFTC were available for this study, a microscale assay was used to study the interaction between these forms of the parasite and macrophages. Macrophage monolayers were set up in sterile slide microwells (3-mm diameter, Carlson Scientific, Peotone, IL) using $2 \cdot 10^4$ nucleated cells. Infection was produced with $2 \cdot 10^4$ MFTC. All other conditions remained as described above.

Myoblast cultures and their infection. Monolayers of rat heart myoblasts (American Type Culture Collection CRL 1446) were prepared in Lab-Tek wells at 37°C in a moist 10% CO_2-in-air atmosphere in DMEM supplemented with 10% fetal bovine serum as described previously [12]. When the cultures attained confluence (approx. 5 days), the medium was removed and 0.4 ml of trypomastigote suspension containing $1 \cdot 10^6$ enzyme-treated or untreated organisms in DMEM-BSA were added. The infected cultures were further incubated under the same conditions for 2 h. At this point in time, these cultures were either interrupted by fixation (see below) or washed to remove non-bound parasites, incubated for an additional 16 h before interruption.

Assay of association of macrophages or myoblasts with T. cruzi. All cultures were terminated by washing the non-bound parasites with MEM (macrophages) or DMEM (myoblasts) and fixation with 2.5% glutaraldehyde in a phosphate-

buffered saline solution for 1 h. After staining with Giemsa, each culture was examined microscopically (1000 ×). Not less than 200 randomly selected cells were screened in each monolayer, recording the number of (a) cells associated with one or more parasites, (b) parasites associated with the cells, and (c) cells not associated with parasites. In this paper, the word association is intended to mean surface bound and internalized parasites. Each test was performed in triplicate and the results were expressed both as the percentage of infacted cells and the number of trypanosomes per cell. All results presented in this paper represent the mean ± S.D.

Treatment of parasites with alpha-mannosidase (mannosidase). Suspensions of BT and AMA in carbohydrate-free Hank's balanced salt solution (HSS) adjusted to pH 5.0 were mixed with mannosidase (specific activity 17 units/mg, Sigma Chemical Co.) so that the reaction mixture contained $4 \cdot 10^7$ organisms and 16 units mannosidase per ml. The mixture was incubated at 25°C for 20 min and then centrifuged at $800 \times g$ for 20 min. After four washings with HSS, the parasites were resuspended in the appropriate medium supplemented with 1% BSA and used for cell culture infection as described above. When MFTC were treated with mannosidase, their concentration was $1.6 \cdot 10^6$ parasites/ml whereas all other conditions remained unchanged. Control assays were set up with parasites which had been mock-treated with HSS alone. The selected concentrations of mannosidase were not toxic for the parasites as determined by measures of viability and motility. When required, mannosidase was inactivated by heating at 100°C for 20 min. All batches of mannosidase used in this work were tested by measurement of the release of *p*-nitrophenol from *p*-nitrophenol-alpha-D-mannoside [13] and found to be active. Evidence for the release of mannose from BT or AMA treated with mannosidase during the 20-min incubation period was obtained by gas chromatography [14] of the supernatant following removal of enzymes by precipitation by heating at 100°C for 5 min [13] and formation of the alditol acetate derivative of mannose [14]. The average amounts of mannose released from $1 \cdot 10^8$ BT and AMA under these conditions was 600 and 340 ng, respectively. No mannose was detected in control tests with either untreated parasites or mannosidase alone incubated for 20 min. The binding of active mannosidase to the parasite was ruled out by the absence of enzymatic activity of mannosidase-treated parasites on the *p*-nitrophenol-alpha-D-mannoside substrate [13] after four washings.

Test of enzymatic activities contaminating mannosidase preparations. The mannosidase preparations used in this work are known to be contaminated with < 0.05% beta-galactosidase, beta-*N*-acetylglucosaminidase, alpha-galactosidase and alpha-L-fucosidase. All of the enzymes were purchased from Sigma Chemical Co. and tested at the contaminating concentration under the same conditions as used for the mannosidase treatment of *T. cruzi*. In addition, mannosidase treatment of the parasite was carried out in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (0.063 mM) and pepstatin A (0.032 mM) (Sigma Chemical Co.). These concentrations were selected because preliminary titrations showed that they were the highest levels lacking toxicity for the parasites.

Treatment of macrophages and myoblasts with mannosidase. Lab-Tek culture chambers containing monolayers of macrophages and myoblasts received 0.4 ml of HSS containing 3.2 and 2.0 units of mannosidase, respectively, and were incubated at 25°C for 20 min. These concentrations of mannosidase were selected because preliminary tests showed that they were not cytotoxic and adequate to exert enzymatic activity on the cells.

Presentation of results and statistical analysis. The results presented in the tables and figure of this paper are typically representative of two or more experiments with the same protocol. Differences between mean values were considered to be significant if $p < 0.05$ as determined by the Mann-Whitney '*U*' test.

Results

Enhanced internalization of mannosidase-treated T. cruzi

Pre-treatment of virulent BT with mannosidase markedly enhanced the ability of the parasites to associate with macrophages as evidenced by increases in both the percentage of infected macro-

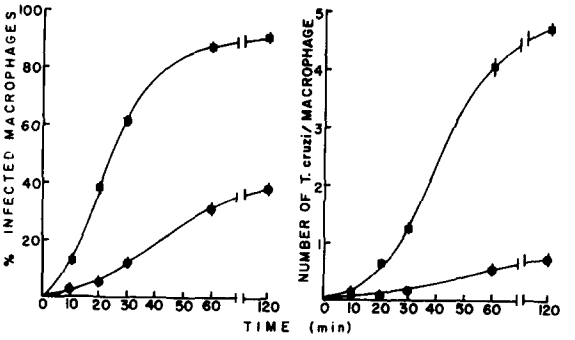

Fig. 1. Effect of mannosidase treatment of BT forms of *T. cruzi* on the kinetics of their association with mouse peritoneal macrophages. Mannosidase-treated BT (■); untreated BT (●). Points represent the mean of triplicate values and vertical lines the standard deviation.

phages and the number of trypanosomes per cell (Fig. 1). Although the effect was detectable as early as 10 to 20 min after exposure of the cells to the parasites, maximal differences between mannosidase-treated and medium-treated parasites were recorded after 2 h of interaction. This was the selected time of interaction for subsequent experiments. The enhancing effect was reproduced when either AMA and MFTC forms of the *T. cruzi* were subjected to the mannosidase treatment (Table I). When myoblasts were used as host cells, their infection by mannosidase-treated BT was also significantly enhanced.

TABLE II

REVERSIBILITY OF THE EFFECT OF MANNOSIDASE TREATMENT OF *T. CRUZI* ON PARASITE INTERACTION WITH MACROPHAGES

The parasites were incubated with mannosidase as described under Materials and Methods. After four washings with HSS, the flagellates were incubated for the indicated periods of time in fresh MEM-BSA and then added to the macrophage cultures. All differences between values obtained with mannosidase-treated parasites and the corresponding control values were statistically significant ($p < 0.05$).

Treatment of BT	Time (h)	% Infacted macrophages	Number of parasites per macrophage
Medium alone	0	39.7 ± 1.7	0.56 ± 0.02
Mannosidase	0	91.3 ± 1.8	4.80 ± 0.14
Mannosidase	1	55.7 ± 0.8	0.73 ± 0.01
Mannosidase	2	44.3 ± 1.8	0.74 ± 0.03

TABLE I

EFFECTS OF MANNOSIDASE TREATMENT OF DIFFERENT FORMS OF *T. CRUZI* ON THEIR INTERACTION WITH MACROPHAGES OR MYOBLASTS

In Expts. 1–3 and 5, all differences between experimental and the corresponding control (no treatment) values were statistically significant ($p < 0.05$). In Expt. 4, the differences were not statistically significant. The term glycosidases refers to a mixture of beta-galactosidase, beta-*N*-acetylglucosaminidase, alpha-galactosidase and alpha-L-fucosidase, each at a concentration equivalent to 0.05% of the mannosidase activity used in Expts. No. 1–3 and 5.

Expt. No.	Treatment	Parasite form	Host cell	% Infected cells	Number of parasites per cell
1	None	BT	Macrophage	41.5 ± 0.04	0.59 ± 0.04
	Mannosidase	BT	Macrophage	97.0 ± 0.7	4.87 ± 0.01
	None	AMA	Macrophage	29.7 ± 5.3	0.63 ± 0.2
	Mannosidase	AMA	Macrophage	53.7 ± 4.9	1.56 ± 0.1
2	None	MFTC	Macrophage	4.7 ± 0.3	0.05 ± 0.0
	Mannosidase	MFTC	Macrophage	17.5 ± 1.3	0.32 ± 0.01
3	None	BT	Myoblast	31.3 ± 0.4	0.91 ± 0.02
	Mannosidase	BT	Myoblast	51.5 ± 0.7	1.86 ± 0.02
4	None	BT	Macrophage	41.6 ± 0.1	0.58 ± 0.02
	Glycosidases	BT	Macrophage	41.3 ± 0.4	0.56 ± 0.03
5	None	BT	Macrophage	41.4 ± 0.2	0.57 ± 0.02
	Mannosidase	BT	Macrophage	97.8 ± 0.04	4.85 ± 0.03
	Mannosidase + PMSF + PA	BT	Macrophage	97.2 ± 0.3	4.85 ± 0.07

TABLE III

EFFECTS OF MANNOSIDASE TREATMENT OF MACROPHAGES OR MYOBLASTS ON THEIR INTERACTION WITH DIFFERENT FORMS OF *T. CRUZI*

HI-mannosidase, mannosidase inactivated by heating at 100°C for 20 min. Only the differences between the values obtained with mannosidase-treated cells and the corresponding control values were statistically significant ($p < 0.05$).

Expt. No.	Treatment	Parasite form	Host cell	% Infected cells	Number of parasites per cell
1	None	BT	Macrophage	41.4 ± 0.2	0.70 ± 0.10
	Mannosidase	BT	Macrophage	16.4 ± 2.2	0.20 ± 0.02
	HI-mannosidase	BT	Macrophage	41.6 ± 2.1	0.67 ± 0.04
	None	AMA	Macrophage	28.3 ± 2.2	0.62 ± 0.02
	Mannosidase	AMA	Macrophage	15.2 ± 1.1	0.03 ± 0.00
	HI-mannosidase	AMA	Macrophage	28.8 ± 0.6	0.63 ± 0.03
2	None	BT	Myoblast	31.2 ± 1.7	0.90 ± 0.06
	Mannosidase	BT	Myoblast	22.3 ± 0.3	0.55 ± 0.01

Since the mannosidase preparations used in this work were contaminated with < 0.05% beta-galactosidase, beta-*N*-acetylglucosaminidase, alpha-galactosidase and alpha-L-fucosidase, the possibility that these enzymes rather than mannosidase were responsible for the noted effects on cell infection was examined. None of these enzymes had any detectable effect on cell-parasite interaction when tested at concentrations identical with those present during the standard mannosidase treatment (Table I). To rule out a possible role by protease contaminants the mannosidase treatment of *T. cruzi* was performed in the presence of the protease inhibitors PMSF and pepstatin A, failing to alter the magnitude of the enhancing effect of mannosidase over macrophage infection (Table I).

Association of BT with macrophages was still enhanced 1 and 2 h after incubation of mannosidase-treated parasites in fresh medium. However, the magnitude of the effect was not as large as when measured immediately after removing the enzyme (Table II).

Inhibition of parasite association following treatment of host cells with mannosidase

Treatment of either macrophages or myoblasts with mannosidase resulted in significant reduction of the association of these cells with *T. cruzi* (Table III). This observation applied to macrophages interacting with either BT or AMA as well as to myoblasts interacting with BT. Both the percentage of infected cells and the number of parasites per cell were affected.

Discussion

These results show that mannose residues present on the surface of both *T. cruzi* and two of its host cells are relevant to the mechanisms of parasite-cell association. Whereas mannosidase treatment of the parasites systematically resulted in increased association with the host cells, similar treatment of the latter had the opposite effect. This contrast highlighted two distinct roles for the membrane mannose residues present on the parasite and its host cells.

Enhanced cell association of mannosidase-treated flagellates was observed when either phagocytic or non-phagocytic cells were used, suggesting that the effect was not a mere consequence of enhanced phagocytosis of the altered parasites. Although BT and MFTC are both infective trypomastigote forms, they are morphologically different and represent two distinct stages in the life cycle of the parasite; the latter being the one transmitted by the insect vectors. However, mannosidase treatment had a comparable effect on the ability of these forms to associate with macrophages, suggesting the existence of similar, if not the same, steps in the mechanisms of association of these forms with host cells. Other investigators have reported that presence of mannose in the

medium does not affect the levels of *T. cruzi* infection of bovine embryo skeletal muscle cells [15]. However, this observation is not necessarily at odds with our own because mannose bound to surface components may affect the conformation or exposure of parasite ligands in ways that free mannose could not.

The effect of mannosidase on BT association with macrophages subsided considerably 1 h after the treatment, emphasizing the reversible nature of the alteration. This observation is consistent with a capacity of the trypanosome to rapidly re-express a membrane component that facilitates its attachment to, and/or its infection of host cells. It should be noted that in these reversibility experiments there was no appreciable transformation of the BT into other forms.

Recent experiments in our laboratory have shown that AMA forms of *T. cruzi* do not infect myoblasts under the conditions used in the present work (Villalta, F. and Kierszenbaum, F., unpublished results), suggesting that they are not invasive. Therefore, association of these forms with macrophages (Table I) probably reflects a phagocytic process. Removal of mannose residues from AMA also increased association of these forms with macrophages, suggesting that mannose residues also play a role in phagocytic uptake of this form. However, the extent of the increase of the number of AMA per macrophage (2- to 3-fold) was much smaller than the increases in BT (more than 8-fold) or MFTC (more than 6-fold) association with the same cells. The differences were not as marked when the percentages of cells infected by BT, AMA and MFTC were compared (Table I), suggesting that different phenomena were being monitored – namely phagocytosis plus cell penetration in the case of the BT and MFTC but only phagocytosis in the case of the AMA. Alternatively, different mannose-containing components may be involved in the association of the various forms of the parasite with macrophages. It should be noted that differences between AMA and trypomastigote forms of *T. cruzi* with respect to phagocytosis have been reported [16].

Mannose could be related to a surface receptor for *T. cruzi* on the macrophages and myoblasts since significant reductions in their association with the parasites were seen when the cells were treated with mannosidase. This inference is consistent with previous findings showing the lectin concanavalin A (whose specific sugar-binding moieties are D-mannose and alpha-D-glucose) inhibits association of macrophages [17] and fibroblasts [18] with trypomastigote forms of blood and tissue culture origin, respectively. Alternatively, mannose might not be a part of the host cell receptor but be sufficiently close to it for its removal to cause topographical alterations, affecting binding with the parasite.

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