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HOST-CELL INVASION BY *Trypanosoma cruzi*: ROLE OF CELL SURFACE GALACTOSE RESIDUES

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Alpha-galactosidase treatment of blood, insect and intracellular forms of *T. cruzi* enhanced their ability to associate with mouse peritoneal macrophages or rat heart myoblasts as evidenced by significant increases in both the percentage of infected cells and the number of parasites per cell. The magnitude of the enhancement was greater with invasive (blood and insect) than with noninvasive (intracellular) forms of the parasite. The enzyme effect was reversible, attaining total recovery in 2.5 hr. By contrast, when either host cell was pretreated with the enzyme, the extent of cell-parasite association was significantly reduced. These results indicate that galactose residues on *T. cruzi* and host cells modulate their association in opposite ways.

Trypanosoma cruzi, the etiologic agent of Chagas' disease, requires an intracellular localization for multiplication in mammalian hosts. Although the attachment of the parasite to host cells is the first step of the internalization process, our understanding of this process is very limited. Existing information indicates that ongoing protein synthesis in the parasite is required for it to associate effectively with some types of host cells (1, 2). It is also known that the balance between intracellular levels of cyclic nucleotides plays an important role in modulating macrophage association with *T. cruzi*, cyclic GMP promoting it and cyclic AMP inhibiting it (3, 4). Some *T. cruzi* surface glycoproteins are thought to play a role in parasite association with some cells (5, 6). Furthermore, mannose and other sugars present on the parasite surface participate in this association in an as yet undefined manner (7-9). Galactomannan (10-11), glycoproteins (12) and phosphoglycoproteins (13) have been identified as sources of galactose (Gal) in culture forms of *T. cruzi*, and this sugar is also present on the surface of the invasive blood forms (BF) (14). To find out if surface Gal partici-

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pates in cell-T. cruzi association, the effects of its removal from the surface of host cells or from different forms of the parasite were studied.

MATERIALS AND METHODS

The Tulahuén strain T. cruzi was used in this work. BF were isolated from the blood of infected mice as described previously (15, 16). Amastigote forms (AMA) were grown in ML15-HA medium (17). Insect-derived, metacyclic forms (IM) were isolated from the hindgut of Rhodnius prolixus infected 4 to 5 weeks previously (16).

To study interactions between untreated or enzyme-treated IM, BF and AMA with macrophages, suspensions of IM were adjusted to 1 X 10^6 organisms/ml whereas BF or AMA were adjusted to 1 X 10^7 organisms/ml. Use of a smaller concentration of IM owed to the relatively low numbers of these organisms obtained from the insects. Parasites to be added to macrophage and rat heart myoblast (RHM) cultures were suspended in Eagle's minimal essential medium (MEM, Gibco, Grand Island, N.Y.) and Dulbecco's modified MEM (DMEM, Gibco), respectively, supplemented with 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis. MO) and antibiotics. These media will be referred to as MEM-BSA and DMEM-BSA, respectively.

The method to measure association of T. cruzi with peritoneal macrophages has been described in detail (9). These cells were obtained from unelicited inbred CBA/J mice (Jackson Laboratory, Bar Harbor, ME). The initial parasite:cell ratio was 4:1. A micro-scale version of the assay system was used when IM were used and was performed in the 3-mm-diameter wells of sterile Teflon-coated microscope slides (Cel-Line, Newfield, N.J.); the initial IM:macrophage was 1:1. After removal of the non-bound organisms, the macrophages were fixed with absolute methanol and stained with Giemsa. Each experimental and control condition was set up in triplicate. Confluent (5-day-old) monolayers of RHM were prepared in Lab-Tek chambers as described elsewhere (1). The cultures were covered with 0.4 ml of BF suspension containing 1 X 10^6 enzyme-treated (see below) parasites/ml and incubated at 37°C for 2 hr in a 10% CO_2-in-air atmosphere. The co-cultures were interrupted as described above except that glutaraldehyde was used as the fixative. Not less than 200 randomly selected cells were microscopically screened in each culture. The numbers of cells free of parasites and of cells associated with (i.e., bound to and internalizing) one or more organisms were counted as well as the total number or parasites. Results were expressed as the percentage of infected cells and the average number of parasites per cell.

A preparation of alpha-D-galactoside galactohydrolase (Galase, EC3.2.1.22, specific activity 9 units/mg protein, Sigma Chemical Co.) from green coffee beans was used to treat the different forms of T. cruzi. Two-tenth ml of BF or AMA suspension at 1 X 10^8 organisms/ml in carbohydrate-free Hank's balanced salt solution (HBSS) pH 6.5 was incubated with 0.1 ml of Galase solution (44.1 units/ml) and 0.2 ml HBSS at 26°C for 20 min. When IM were treated with the enzyme, the parasite suspension contained 1 X 10^6 IM/ml and all other conditions were kept the same. After washing four times with HBSS, the parasites were resuspended in MEM-BSA or DMEM-BSA at the concentrations described above. Parasites used in control assays were subjected to all of these treatments except that enzyme-free HBSS was used. The selected concentration of Galase was not toxic for the parasites since it did not affect motility and viability. When required, Galase was inactivated by heating at 100°C for 20 min. All batches of Galase used in this work were active as evidenced by the release of p-nitrophenol from p-nitrophenol-alpha-D-galactoside (18).

Evidence for the release of Gal from parasites treated with the enzyme during the 20-min incubation period was obtained by gas chromatography (19) of the supernatant following removal of the enzyme by precipitation by heating at 100°C for 5 min (20) and formation of the alditol acetate derivative of Gal

(19). Control tests were performed with the supernatants of suspensions of untreated parasites and a solution of Galase alone incubated for 20 min. Parasites were also treated with Galase as above after substituting 0.2 ml of melibiose (6-O-alpha-D-galactopyranosyl-D-glucose) solution in HBSS for the carbohydrate-free HBSS. The final concentration of melibiose was 0.54M. In this case, the solutions of melibiose and Galase were mixed together immediately before addition of the parasites. The substrate specificity of melibiose for Galase has been documented (21). At the selected concentration, melibiose had no detectable effect of parasite motility or viability.

To monitor the kinetics of cell-parasite association after Galase treatment of T. cruzi, cells and parasites were exposed to each other for varying periods of time starting immediately after removing the enzyme. To establish the kinetics of recovery from the Galase treatment, the parasites were washed and incubated at 37°C for varying periods of time before used.

The parasites were also treated with enzymes known to contaminate the Galase preparation [alpha-glucosidase, alpha-mannosidase and beta-N-acetyl-glucosaminidase (Sigma Chemical Co.)] at the contaminating concentrations (0.0023%, 0,052% and 0.034%, respectively). In addition, Galase treatment of the parasite was carried out in the presence of the protease inhibitors phenylmethyl-sulfonyl fluoride (PMSF, 0.063mM) and pepstatin A (PA, 0.032mM) (Sigma Chemical Co.). These concentrations were selected because preliminary titrations showed them to be the highest lacking toxicity for the parasites.

Macrophage and RHM were treated with 22 and 5.6 units Galase/ml, respectively at 26°C for 20 min. These concentrations were not cytotoxic but adequate to exert enzymatic activity on the cells. The parasites were added to the cultures after washing off the enzyme with HBSS.

All sets of results presented in the tables and figure of this paper are typically representative of the two to five repeat experiments. Differences were considered to be significant if $P<0.05$ by Student's "t"-test.

RESULTS

After treatment with Galase, BF showed an enhanced ability to associate with macrophages as evidenced by significant ($P<0.05$) increases in both the percentage of infected cells and the number of organisms per cell (Fig. 1). The effect was noted as early as 10 min after exposure of the cells to the parasites and attained a much greater proportion after 2 hr. The average amount of Gal released by Galase from BF during the 20-min incubation period was 7.7 µg Gal/1 X 10^{10} organisms. No Gal was detected in control tests performed with either untreated parasites or Galase alone incubated for 20 min. Binding of active Galase to the parasite was ruled out by the absence of enzymatic activity of Galase-treated parasites on the p-nitrophenol-alpha-D-galactoside substrate after four washings (data not shown). An enhancing effect of Galase treatment on parasite-cell association was also seen when RHM were used as host cells (Fig. 1) and when either AMA or IM were treated with the enzyme (Table 1). The degree of enhancement was greater with the infec-

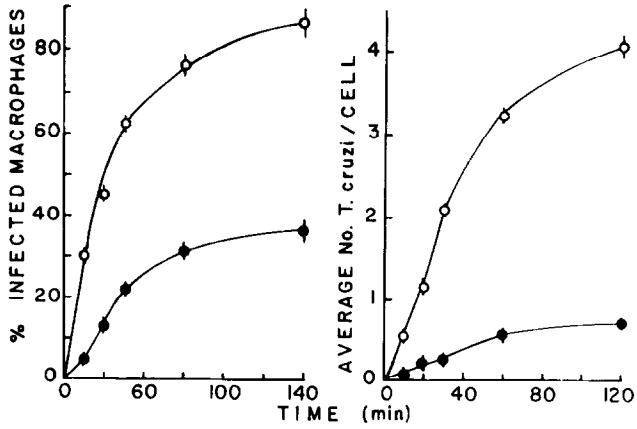

Fig. 1 Effects of pretreatment of BF forms of T. cruzi with Galase on the kinetics of parasite association with macrophages. Abscissa, time of incubation of enzyme-treated (o---o) or HBSS-treated (•---•) trypanosomes with untreated macrophages. Parasites were added to the macrophage cultures immediately after removal of the enzyme or a comparable mock treatment with HBSS. Points represent the mean of triplicate determinations and vertical lines the standard deviation.

tive BF and IM than with the non-invasive AMA. When T. cruzi was treated with Galase in the presence of melibiose, the degree of enhancement of macrophage-parasite association was significantly decreased. Treatment of the trypanosomes with melibiose alone had no detectable consequence.

Since the Galase preparations were contaminated with minute amounts of alpha-glucosidase, alpha-mannosidase and beta-N-acetylglucosaminidase (see Materials and Methods), these enzymes were tested for their ability to affect BF-macrophage association at the same concentrations as present in the Galase treatment reaction mixtures. None of these enzymes had any significant consequence on BF-cell interaction[1] (data not shown). Furthermore, presence of two protease inhibitors, PMSF and PA, during the Galase treatment failed to alter the enhancing effect of the enzyme (Table 1). The Galase effect on BF association with host cells subsided significantly 1 hr after removing the enzyme and was no longer detectable after 2 to 2.5 hr (Table 2).

The degrees of association of macrophages or RHM pretreated with Galase with either BF or AMA were significantly smaller than those exhibited by

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[1] These results should not be construed as representing that these enzymes would not have an effect on parasite-cell association at higher concentrations.

TABLE 1

EFFECT OF Galase PRETREATMENT OF BF, AMA AND IM FORMS OF *T. cruzi* ON THEIR ASSOCIATION WITH MACROPHAGES AND RHM

Exp. No.	Pretreatment	Parasite form	Host cell	% Infected cells	No.parasites/cell
1	HBSS	BF	Macrophage	36.4 ± 0.6	0.68 ± 0.01
	Galase	BF	Macrophage	87.6 ± 0.5	4.39 ± 0.27
	HBSS	AMA	Macrophage	30.1 ± 0.0	0.61 ± 0.06
	Galase	AMA	Macrophage	58.3 ± 1.3	2.31 ± 0.01
2	HBSS	IM	Macrophage	5.0 ± 0.3	0.05 ± 0.01
	Galase	IM	Macrophage	22.9 ± 0.9	0.49 ± 0.04
3	HBSS	BF	Macrophage	36.6 ± 0.2	0.68 ± 0.01
	Galase	BF	Macrophage	87.6 ± 0.4	4.37 ± 0.28
	Galase+PMSF+PA	BF	Macrophage	87.5 ± 1.4	4.40 ± 0.42
4	HBSS	BF	RHM	26.2 ± 1.3	0.31 ± 0.04
	Galase	BF	RHM	48.8 ± 0.4	0.79 ± 0.06
5	HBSS	BF	Macrophage	27.7 ± 1.3	0.37 ± 0.04
	Galase	BF	Macrophage	86.2 ± 0.6	4.98 ± 0.07
	Galase+Melibiose	BF	Macrophage	49.4 ± 1.4	1.33 ± 0.24
	Melibiose	BF	Macrophage	28.5 ± 1.2	0.40 ± 0.02

In all tables, the results are expressed as the mean of triplicate determinations ± SD. All differences between results obtained with Galase-treated and mock-treated parasites or cells were statistically significant. The difference between the effects of the Galase treatments performed in the presence and absence of melibiose (Exp. No. 5) was also statistically significant.

untreated host cells (Table 3). Treatment of parasites, macrophages or RHM with heat-inactivated Galase had no significant consequence on any of the cell-parasite interactions examined in this work.

DISCUSSION

The roles played by Gal residues on *T. cruzi* and host cells were clearly not the same since Galase treatment of the former systematically

TABLE 2

REVERSIBILITY OF THE Galase EFFECT ON BF OF *T. cruzi* ON THEIR ASSOCIATION WITH MACROPHAGES

Pretreatment of BF	Time (hr)	% Infected macrophages	No.parasites/macrophage
HBSS	0	32.4 ± 0.9	0.55 ± 0.05
Galase	0	86.3 ± 1.0	3.79 ± 0.06
Galase	1	49.2 ± 0.3	1.01 ± 0.08
Galase	2	36.8 ± 0.3	0.68 ± 0.01
Galase	2.5	32.4 ± 0.3	0.56 ± 0.01

TABLE 3

EFFECTS OF Galase TREATMENT OF MACROPHAGES OR RHM ON THEIR INTERACTION WITH DIFFERENT FORMS OF T. cruzi

Exp. No.	Treatment	Parasite form	Host cell	% Infected cells	No.parasites/cell
1	HBSS	BF	Macrophage	34.6 ± 0.9	0.62 ± 0.09
	Galase	BF	Macrophage	13.0 ± 0.0	0.22 ± 0.03
	HI-Galase	BF	Macrophage	34.6 ± 1.5	0.62 ± 0.03
	HBSS	AMA	Macrophage	47.8 ± 0.6	0.91 ± 0.02
	Galase	AMA	Macrophage	25.2 ± 0.2	0.01 ± 0.03
	HI-Galase	AMA	Macrophage	47.5 ± 0.4	0.93 ± 0.01
2	HBSS	BF	RHM	25.9 ± 0.6	0.32 ± 0.04
	Galase	BF	RHM	9.5 ± 2.1	0.11 ± 0.04

Only the differences between the values obtained with Galase-treated cells and the corresponding control values were statistically significant ($P<0.05$).

increased their association with macrophages or RHM whereas treatment of the latter yielded opposite results. The effect of pretreatment of the parasites with Galase was qualitatively the same whether a phagocytic or non-phagocytic cell was the host cell, suggesting that the noted enhancement was not merely due to facilitated phagocytosis of parasites.

Several observations indicated that Galase was responsible for the noted effects. Enzymes present in the Galase preparation failed to produce any detectable changes in parasite-cell association at the highest possible contaminating concentration and so did two protease inhibitors. Furthermore, melibiose, a specifically substrate for Galase, effectively competed with the parasite, inhibiting the enhancing effect of the enzyme.

Although BF and IM forms of T. cruzi can penetrate cell membranes, they represent morphologically distinct parasite forms. However, Galase treatment had a comparable effect on their abilities to associate with macrophages, suggesting that similar, if not the same, mechanisms may be involved in their association with host cells. The degree of enhancement of the association between of Galase-treated BF or IM (7- to 10-fold) with untreated macrophages was always greater than that recorded with enzyme-treated AMA (3- to 4-fold). Increased association of macrophages with

BF and IM would be expected to facilitate both phagocytosis of and cell invasion by the organisms whereas increased association with the non-invasive AMA (9) would enhance only phagocytosis. Alternatively, different Gal-containing surface components might be involved in association of the various forms of the parasite with host cells. Differences in macrophage uptake of BF and AMA under otherwise identical conditions have been reported (22).

Pretreatment of either host cells or BF forms of T. cruzi with 50mM Gal has been shown by other workers not to affect the degree of parasite association with bovine embryo skeletal muscle cells (23). These observations, however, are not in disagreement with our results because the presence of Gal in parasite or host cell surface molecules is unlikely to influence the conformation or exposure of these molecules in the same ways as free Gal. Furthermore, removal of Gal residues by Galase treatment would be expected to have more profound consequences on the topography of cell membranes than mere presence of free Gal in the medium.

The inhibitory effect of Galase on BF association with macrophages was greater when tested immediately after removal of the enzyme than after 1 hr of incubation of enzyme-treated organisms in fresh medium (Table II). This observation, together with the fact that the inhibitory effect was no longer detectable after 2 hr, revealed the reversible nature of the alteration(s) caused by the enzyme and suggested a relatively rapid rate of re-expression of surface components involved in cell-parasite attachment. Since transformation of BF into other forms of the parasite was not detectable during these experiments, the smaller degree of enhancement could not have been due to replacement of BF by AMA.

Pretreatment of macrophages or RHM with Galase significantly decreased cell-parasite association (Table III). This observation is in keeping with the results of Henriquez et al. (7), who showed that treatment of several cell types with ricin I (a lectin that specifically binds Gal residues) inhibited their infection by cultured-derived T. cruzi trypomastigote. This implies that Gal could be a building block of a putative cell surface

receptor for invasive forms of T. cruzi. Alternatively, Gal might be a part of an irrelevant molecule whose proximity to the receptor is such that its removal or blocking would alter the topography of the host cell membrane and affect parasite binding.

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